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THE

BIOLOGICAL BULLETIN

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INSTRUCTIONS TO AUTHORS

THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except for a limited number of solicited review papers which may be accepted after formal refereeing), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review.

1. ***Manuscripts.*** Manuscripts, including figures, should be submitted in triplicate. (Xerox copies of photographs are not acceptable for review purposes.) The original manuscript must be typed in double spacing (*including* figure legends, footnotes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. Manuscripts should be proofread carefully and errors corrected legibly in black ink. Pages should be numbered consecutively. Margins on all sides should be at least 1 inch (2.5 cm). Manuscripts should conform to the *Council of Biology Editors Style Manual*, 4th Edition (Council of Biology Editors, 1978) and to American spelling. Unusual abbreviations should be kept to a minimum and should be spelled out on first reference as well as defined in a footnote on the title page. Manuscripts should be divided into the following components: Title page, Abstract (of no more than 200 words), Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited, Tables, and Figure Legends. In addition, authors should supply a list of words and phrases under which the article should be indexed.

2. ***Figures.*** Figures should be no larger than 8½ by 11 inches. The dimensions of the printed page, 5 by 7¾ inches, should be kept in mind in preparing figures for publication. We recommend that figures be about 1½ times the linear dimensions of the final printing desired, and that the ratio of the largest to the smallest letter or number and of the thickest to the thinnest line not exceed 1:1.5. Explanatory matter generally should be included in legends, although axes should always be identified on the illustration itself. Figures should be prepared for reproduction as either line cuts or halftones. Figures to be reproduced as line cuts should be unmounted glossy photographic reproductions or drawn in black ink on white paper, good-quality tracing cloth or plastic, or blue-lined coordinate paper. Those to be reproduced as halftones should be mounted on board, with both designating numbers or letters and scale bars affixed directly to the figures. All figures should be numbered in consecutive order, with no distinction between text and plate figures. The author's name and an arrow indicating orientation should appear on the reverse side of all figures.

3. ***Tables, footnotes, figure legends, etc.*** Authors should follow the style in a recent issue of *The Biological Bulletin* in preparing table headings, figure legends, and the like. Because of the high cost of setting tabular material in type, authors are asked to limit such material as much as possible. Tables,

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COMPARATIVE ANATOMY OF THE TUNICATE TADPOLE, *CIONA INTESTINALIS*

MICHAEL J. KATZ

Marine Biological Laboratory, Woods Hole, MA 02543 and Department of Developmental Genetics and Anatomy, Case Western Reserve University, Cleveland, OH 44106

ABSTRACT

At 19–20 h of age, the *Ciona intestinalis* ascidian tadpole has six developed organ systems (tunic, epidermis, notochord, tail musculature, adhesive organ, and nervous system) and approximately four organ system rudiments (including: primordial pharynx, atrial primordia, gut primordium, and mesodermal pockets). Contemporary light and electron microscopic descriptions of the anatomy and the histology of these tissues support the old contention, originated by Kowalevsky in 1866, that the body plans of tunicate tadpole and of vertebrate larvae are homologues.

INTRODUCTION

In the first volume of *Nature* (1869), there is a note entitled "Kinship of Ascidians and Vertebrates". It reads, in part:

> Prof. Kupffer . . . asserts that his [studies on the embryology of the tunicate *Phallusia*] in large measure agree with those of Kowalevsky touching the startling vertebrate features of the early condition of these invertebrata . . . He says: "At this stage one could not imagine a more beautiful model of a vertebrate embryo, with the neural tube on one side of the axis and a visceral tube on the other." . . . He promises full details shortly, and we hope to be able to return to this most important matter. (Foster, 1896)

Why were the vertebrate features of tunicate tadpoles called "startling"?

The tunicates were known to Aristotle: he called them *Tethya* or *Thalia*, and he was unsure whether they were plants or animals (Berrill, 1950; Singer, 1959; Barrington, 1965). Lamarck (1809), who named the group *Tunicata*, considered them to be molluscs. Cuvier (1828) classified the tunicates as shell-less acephalic molluscs; and likewise all other major biologists of the first half of the nineteenth century categorized tunicates as molluscs (Berrill, 1950). Thus, until the mid 1860s, the tunicates, a numerically significant group of organisms (Millar, 1971) with possibly 2000 extant species (Young, 1962), were thought to be prostomian invertebrates—exceedingly distant from the vertebrate phylogeny.

In 1866, this view changed dramatically when the comparative embryologist A. Kowalevsky discovered that the larval tunicate possesses a dorsal tubular nervous system, a ventral tubular gut, and an axial notochord flanked by muscles. In other words, the tunicate tadpole appears to have a characteristic vertebrate body plan. That the tunicates are actually members of the chordate phylogeny was irrefutably confirmed with the demonstrations by Kowalevsky (1866), Willey (1893a, b), and Conklin (1905) that the "mouth" (the 'pharynx') of the tunicate develops as a secondary opening at the end of the embryo farthest from the blastopore and that during embryogenesis the neural tube of the tunicate develops from the rolling up of a dorsal neural plate.

Received 23 July 1982; accepted 5 November 1982.

Today, the tunicates are classified as urochordates, a subphylum of the chordates. This classification is based almost entirely on the larval anatomy (Berrill, 1950; Young, 1962; Barrington, 1965; Romer, 1970; Plough, 1978). Recent comparative biochemistry is consistent with this classification (Atkin and Ohno, 1967; Ohno, 1970; Schmidtke *et al.*, 1977, 1978; Fisher *et al.*, 1980). After their metamorphosis, most tunicates lose their notochords and their tubular nervous systems (Berrill, 1950; Cloney, 1977a), and the adult ascidian bears little resemblance to a contemporary chordate. ('Ascidian' is the class of sessile tunicates, commonly called the "sea squirts".)

Anatomical studies of the tunicate tadpole were pioneered by Kowalevsky (1866), Willey (1893a, b), Conklin (1905, 1931), Grave (1921), and Garstang (1928). Similar observations have also been reported by Scott (1946), Berrill (1947a, b), Abbott (1955), Trason (1957), and Anderson *et al.* (1976) (and summarized in: Herdman, 1932; Brien, 1948). The only anatomical studies using modern techniques have focussed on a few specific features, such as the sensory receptor cells (*e.g.*, Dilly, 1962, 1964; Barnes, 1971; Eakin and Kuda, 1971), the tail musculature (*e.g.*, Pucci-Minafra, 1965; Cavey and Cloney, 1972; Burighel *et al.*, 1977), and the notochord (Cloney, 1964, 1969). (For other examples, see: Mackie and Bone, 1976; Cloney, 1977b; Cloney and Cavey, 1982.) To interpret the old anatomical reports in the light of modern biological understanding, I have reexamined the anatomy of the tunicate tadpole using contemporary microscopic techniques. I have chosen to study the tadpole of the ascidian *Ciona intestinalis* because it is probably the most generalized of the readily available and well-studied tunicates (Berrill, 1950; Plough, 1978; J. R. Whittaker, personal communication).

MATERIALS AND METHODS

Animals

Adult *Ciona intestinalis* were collected during June and July along the north shore of Cape Cod near Sandwich MA. The animals were maintained under constant light in running sea water at the Marine Biological Laboratory, Woods Hole. *Ciona* tadpoles were obtained by mixing sperm and eggs from three or four adults. The fertilized eggs were washed and then grown at 18°C in finger bowls of filtered sea water. All histology was done on newly hatched tadpoles, 19–20 h after fertilization. See Whittaker (1977) for a timetable of the developmental stages of *Ciona intestinalis* embryos raised at 18°C.

Histological techniques

Tunicate tadpoles were prepared for both light and electron microscopy using the same histological schedule, similar to the procedures described by Cloney and Florey (1968). All histological steps (except uranyl acetate staining) were done at room temperatures. First, the animals were fixed for 1 h in buffered 2.5% glutaraldehyde (2.5% glutaraldehyde in 0.215 M NaCl, 0.174 M Na_2HPO_4, 0.05 M sucrose, and 0.03 M NaH_2PO_4) and postfixed for ½ h in 1% osmium tetroxide. Washes were in a 1150 mOsm sodium phosphate buffer at pH 7.4–7.6. Next, tissues were dehydrated in ethanol, treated with propylene oxide, and then embedded in Epon 812. Thick sections (approx. one micron each) were stained with ½% toluidine blue for light microscopy, and thin sections were stained with 7.5% uranyl acetate (aqueous) for 10 min at 60°C followed by Reynold's lead citrate for 2 min at room temperature. Thin sections were examined at 80kV with a Zeiss EM 10CA transmission electron microscope.

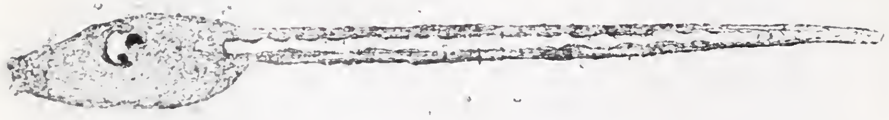

FIGURE 1. Light microscopic photograph of 19–20 h *Ciona intestinalis* tadpole. The outer tunic is covered by adherent inner follicle cells ('test cells'). The tadpole is quite transparent, and the two CNS pigment cells, the dorsal and the ventral melanocytes, can clearly be seen in the ventricle of the prosencephalon. The vacuolated notochord runs prominently down the center of the entire tail. (Photograph courtesy of T. H. Meedel and J. R. Whittaker, Boston University Marine Program, M.B.L., Woods Hole.)

RESULTS

The newly hatched tunicate tadpole is a tiny organism resembling a mammalian spermatozoan. At 19–20 h, *Ciona* tadpoles are uniformly 1.1 mm in length. The body is a tapering cylinder 0.2 mm long and 0.1 mm wide (Fig. 1). The tail is about 0.8 mm long and is 0.03 mm high and 0.02 mm wide at its largest cross-section. The tadpoles are quite transparent, and in living unstained organisms the most striking features are the two black pigment cells, the ventral and the dorsal melanocytes, of the prosencephalon of the central nervous system. With DIC (Nomarski) microscopy, most of the cells of the living tadpole can be distinguished at about 400× magnification. *Ciona* tadpoles swim in fits and starts from the time of hatching, but they do not feed. The earliest metamorphic changes can be detected about 36 h after fertilization.

The tissues of the tunicate tadpole can be usefully divided into two categories: the six developed organ systems (the "larval action-systems" [Grave, 1944; Scott, 1946]), and the four organ system rudiments.

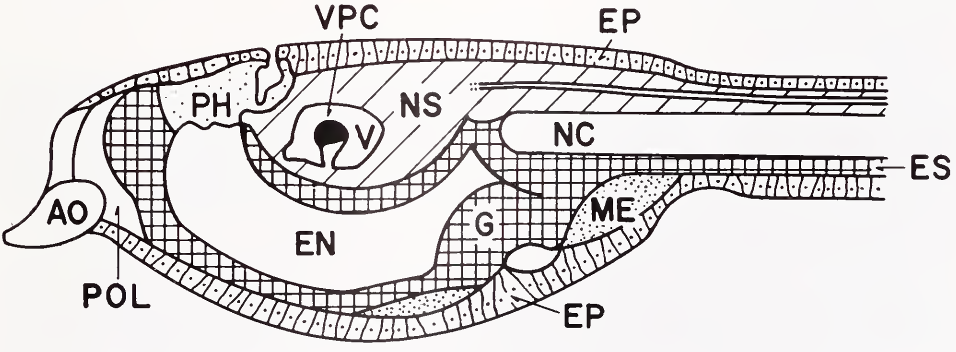

FIGURE 2. Mid sagittal section of 19–20 h *Ciona* tadpole, reconstructed with camera lucida from greater than 100 serial 1 μm plastic cross-sections. AO = adhesive organ, EN = endodermal cavity, EP = epidermis, ES = endodermal strand, G = gut primordium, ME = mesodermal pocket, NC = notochord, NS = nervous system, PH = primordial pharynx, POL = preoral lobe, V = ventricle of prosencephalon, VPC = ventral melanocyte.

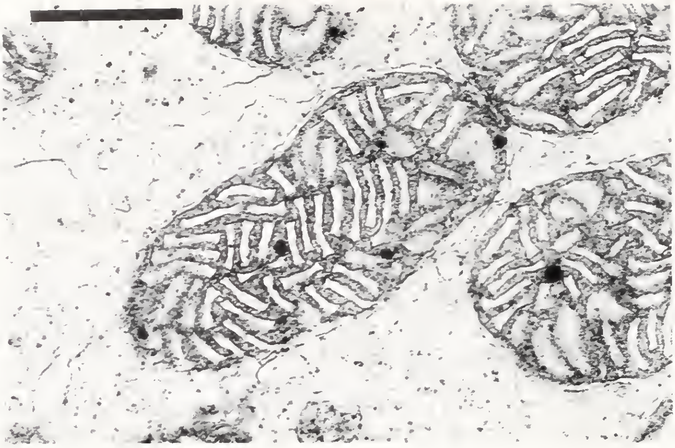

FIGURE 3. Typical mitochondrion of tunicate tadpole (tail musculature, bar = 0.5 μm).

General histology

Cells in the tunicate tadpole tend to be small, less than 10 μm in diameter. The notable exceptions are the muscle cells of the tail which are mononucleated fusiform cells greater than 100 μm in length. All cells of the 19–20 h tadpole contain yolk vesicles, and the highest concentration of yolk vesicles is found in cells of the gut primordium. Yolk vesicles are bounded by a membrane, and internally they appear to be finely granular and homogeneous. Yolk vesicles are roughly spherical and range from about 1.5–3.0 μm in diameter. In general, mitochondria appear to be elliptical or dumb-bell shaped. They range in cross-sectional diameter from 0.4–0.6 μm and in length from 0.6–1.4 μm. All mitochondria have strikingly rectangular cristae and contain dense granules (Fig. 3). Throughout the tadpole, all nuclei appear to have prominent nucleoli. In the following sections, I describe vacuoles in certain cell types—such vacuoles can be the result of poor oxygenation of the animals prior to fixation (B. Tandler, personal communication), and it is possible that they may have been artificially exaggerated in my preparations.

Developed organ systems

1. Tunic

The tunicate tadpole is completely covered by an extracellular "skin" or tunic ('T' in Figs. 4–9) which forms the fins of the animal. The fins all run along the long axis of the animal. The largest fins consist of a dorsal and a ventral flap of tunic running the entire length of the tadpole. One or two major fins also run along each lateral surface of the body. In addition, many tiny fins and short finger-like projections are found along the outer surface of the tunic (Fig. 8).

The tunic itself is acellular. Its outermost edge is formed by a rind (the 'outer cuticle', Figs. 5–7) which is about 40–50 nm thick and which stains densely with a wide variety of EM and LM stains. The innermost edge forms another thinner (10–12 nm thick) rind, the 'inner cuticle' (Fig. 5). A number of highly vacuolated inner follicle cells (also called 'test cells') from the oocyte still adhere to the outer surface of the tadpole tunic (Fig. 1). Along the inner surface of the tunic, the epidermal cells form a generally smooth boundary, and cell processes are rarely found

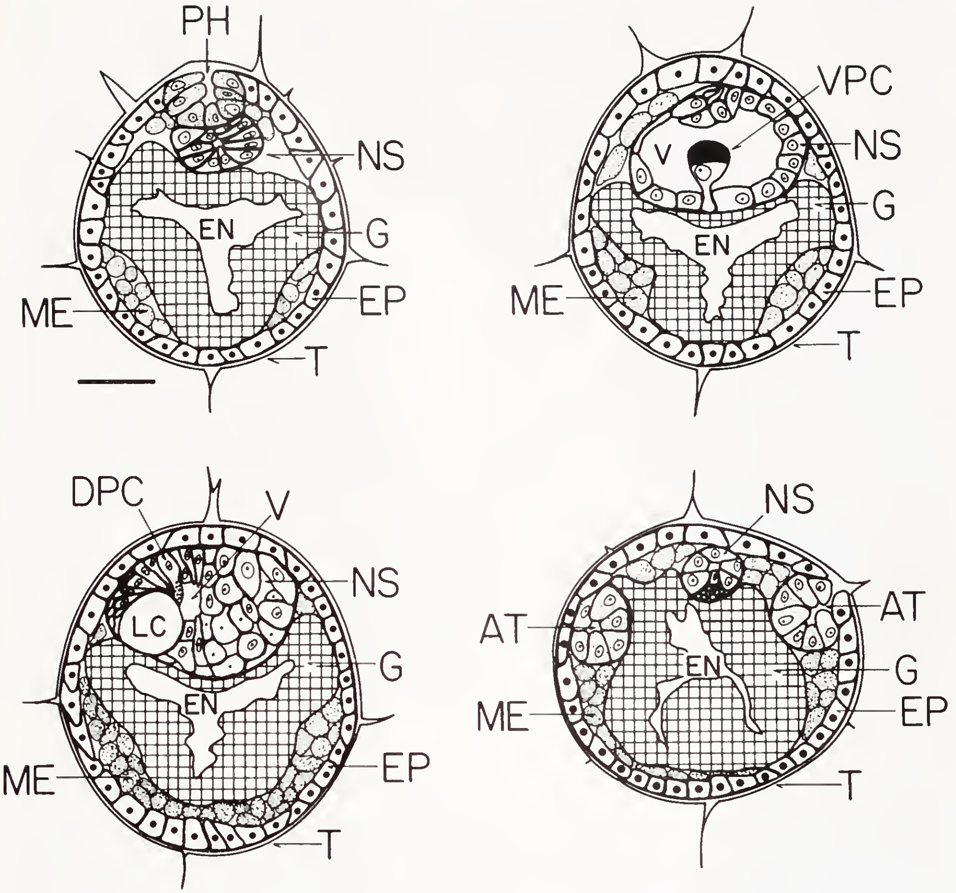

FIGURE 4. Camera lucida tracings of four 1 μm plastic cross-sections through the body of a 19–20 h *Ciona* tadpole. Proceeding rostrally to caudally: upper left = level of primoridial pharynx, upper right = level of ventral melanocyte, lower left = level of ocellus, lower right = level of atria. AT = atrial primordium, DPC = dorsal melanocyte, EN = endodermal cavity, EP = epidermis, G = gut primordium, ME = mesodermal pocket, NS = nervous system, PH = primordial pharynx, T = tunic, V = ventricle of prosencephalon, VPC = ventral melanocyte. Bar = 10 μm.

protruding into the tunic. There are, however, occasional epidermal cilia. (See description of epidermis, below, for more details.) Recent ultrastructural studies have examined the tunic and its development in detail and have described an irregular meshwork of filaments throughout the tunic (Dilly, 1969a; Mancuso, 1973, 1974; Gianguzza and Dolcemascolo, 1980; Cloney and Cavey, 1982).

2. Epidermis

A single-layered epidermis covers the entire animal (Figs. 2, 4–9, 11) except where the pharynx and the two atria break through. The combination of a single-layered epidermis and an outer tunic-like secretion (probably derived from the epidermal cells [Gianguzza and Dolcemascolo, 1980]) is more characteristic of invertebrate integument than of vertebrate integument (Spearman, 1973—see also Wellings and Brown, 1969). In the tunicate tadpole, the epidermal cells form a simple

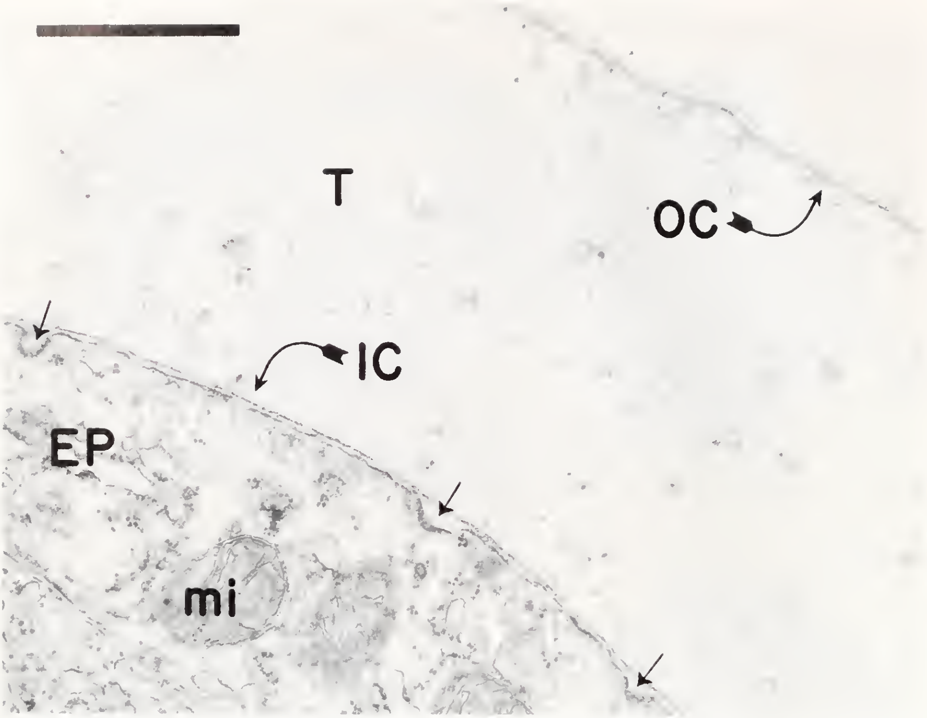

FIGURE 5. Electron micrograph of outer edge of epidermis in the tadpole body. Arrows point to coated vesicles in various stages of formation. EP = epidermal cell, IC = inner cuticle, mi = mitochondrion, OC = outer cuticle, T = tunic. Bar = 1 μm.

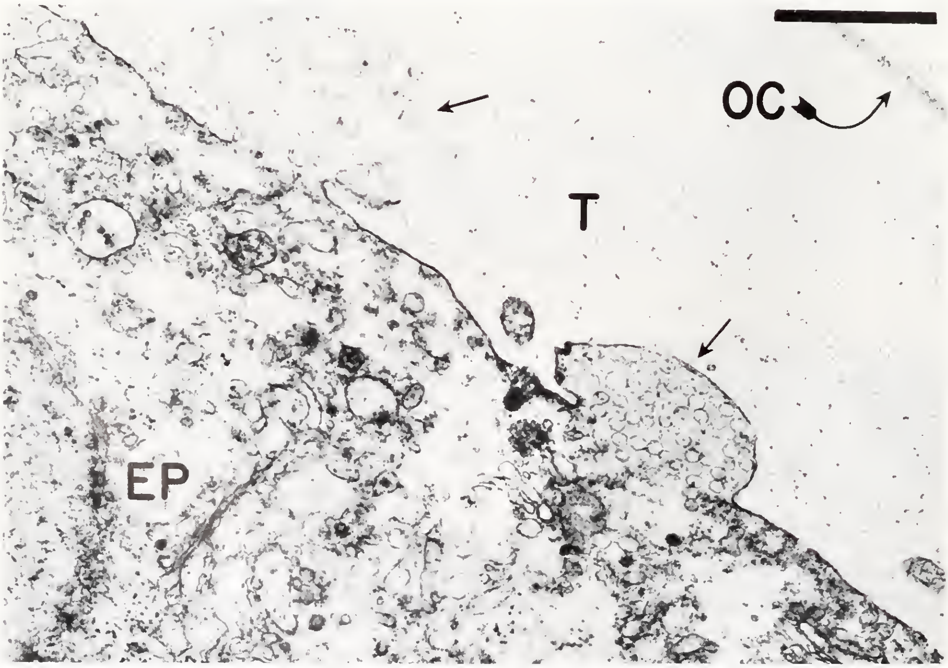

FIGURE 6. Electron micrograph of outer edge of epidermis in the tadpole tail. Arrows point to vesicles in two stages of exocytosis. EP = epidermal cell, OC = outer cuticle, T = tunic. Bar = 1 μm.

squamous or cuboidal epithelium. The thickest cells are about 9 μm from base to apex; these are found both dorsally and ventrally in the tail. At its thinnest (along the lateral walls of the tail), the epidermis is made of flattened cell processes only about 1 μm thick (Figs. 7, 8).

Throughout the epidermis, the outer cell surface is generally smooth without microvilli or extensive ridges. Notable exceptions are finger-like non-ciliated cell processes that extend as far as 2.5 μm into some of the longer fins of the tunic. In addition, as Dilly (1969a) has reported, occasional cilia (with a 9 + 2 microtubule arrangement) protrude from the outer edge of some epidermal cells. The external surface of the epidermis appears quite active, being studded with coated pits in all stages of formation (Fig. 5), especially along the body epidermis. The external surface also contains exocytotic vesicles, especially along the tail epidermis (Fig. 6). The boundaries between adjacent epidermal cells tend to be fairly smooth, although cells do interdigitate to some extent with their neighbors. Mackie and Bone (1976) and Georges (1979) have described gap and tight junctions but no septate junctions in the epidermises of a variety of tunicate tadpoles. Dilly (1969a) suggested the possibility of some desmosomes in the *Ciona* tadpole epidermis. (See Green and Bergquist [1982] for a review of the phylogeny of intercellular junctions.) The inner surfaces of the epidermal cells are fairly smooth and sit upon a basal lamina, which ranges in thickness from 40–120 nm. There is no underlying dermis.

The epidermal cells have large oval nuclei (3 × 4 μm in diameter). Some cross-sections show two nucleoli per nucleus, and this may be the standard complement for epidermal cells. Occasionally, the cells contain large (2–4 μm in diameter) vacuoles. The epidermal cells are not filled with special non-yolk vesicles, as they are in some related animals (*e.g.*, *Amphioxus* [Olsson, 1961]).

3. Notochord

Chordate larvae are characterized by a prominent notochord, which in anamniotes can be larger in diameter than the spinal cord (Goodrich, 1930; Neal and Rand, 1936; Leeson and Leeson, 1958; Bruns and Gross, 1970; Ruggeri, 1972; Jurand, 1974). In *Ciona* tadpoles, the notochord is a rod-shaped tissue, approximately 0.7 mm long and 9–10 μm in diameter, running the length of the tail and extending into the caudal body (Figs. 2, 7, 8, 10, 14). Dorsally, the notochord is covered by the deuterencephalon of the nervous system; ventrally, it overlies the endodermal strand; and laterally, it is surrounded by the muscles of the tail. The development of the notochord of *Ciona* has been well described by Conklin (1905), Cloney (1964), and Mancuso and Dolcemascolo (1977).

The notochord is composed of about 40 cells aligned longitudinally in single file. By the time of hatching, the notochord cells have become a simple squamous epithelium surrounding a matrix-filled lumen, with much the same geometry as a vertebrate endothelial capillary (Fig. 8). The anterior end of the notochord is capped by a single notochord cell. The basal surfaces of the notochord cells rest on a basal lamina that completely surrounds the notochord and that is termed the 'notochordal sheath'. The notochordal sheath ranges from 60–175 nm in thickness. It contains many circumferentially oriented and longitudinally oriented filaments (Cloney, 1964, 1969)—these extracellular fibers are arranged orthogonally, as is the case for certain vertebrate notochordal sheaths (*e.g.*, *Xenopus* tadpoles, M. J. Katz, unpublished observations). (For descriptions of the notochordal sheaths of other vertebrates, see: Leeson and Leeson, 1958; Jurand, 1962, 1974; Waddington and Perry, 1962; Bruns and Gross, 1970. Mathews [1967] provides a review of the evolution of connective tissue macromolecules, such as those forming the notochordal sheaths.)

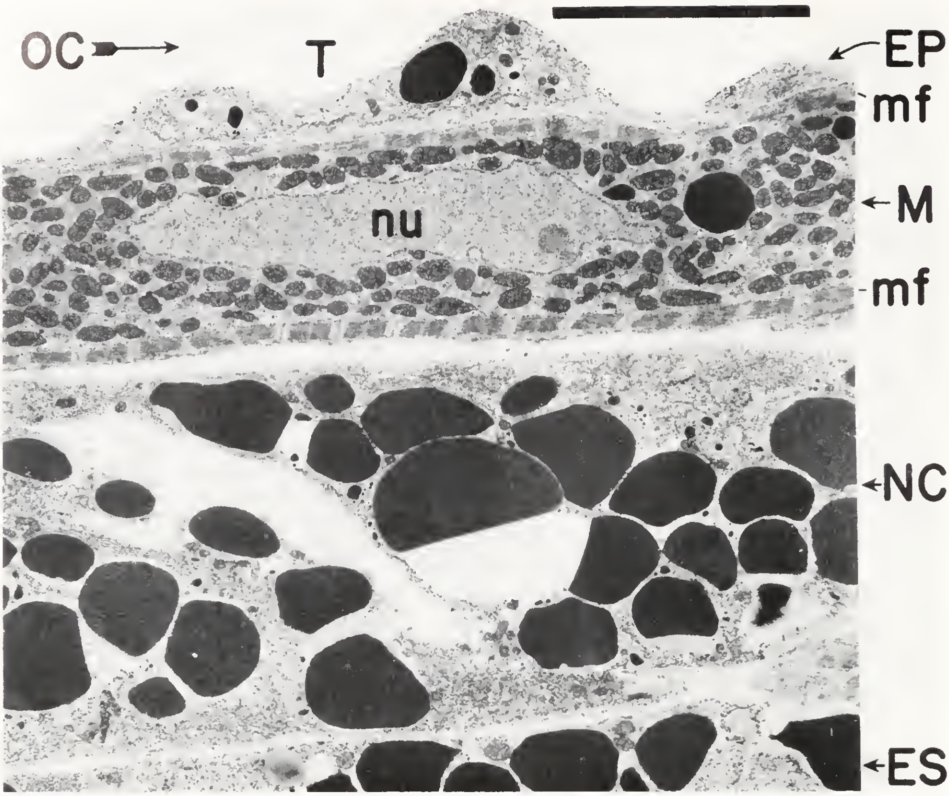

FIGURE 7. Electron micrograph of oblique longitudinal section through the tail. EP = epidermis, ES = endodermal strand, M = muscle cell, mf = myofibrils, NC = notochord, nu = nucleus of muscle cell, OC = outer cuticle, T = tunic. Bar = 1 μm.

The notochord cells themselves have irregularly shaped nuclei, which are about 4 μm in diameter. The cytoplasm is dark and quite granular, and the cells contain much moderately dilated rough endoplasmic reticulum (RER). The notochord cells also contain prominent yolk vesicles. More numerous than the yolk vesicles are other slightly larger (2.5–4 μm in diameter) membrane-bound vesicles that are filled with less densely packed material (Fig. 14). Occasionally, these vesicles can be seen budding off from the cell surface and appearing to empty their contents into the notochordal lumen ('star' in Fig. 8). In the notochordal cells, mitochondria are much more sparse than in the surrounding muscle and nervous system cells. The notochord cells of *Ciona* do not contain the thick bundles of intracellular filaments that are found in the notochord cells of *Amphioxus* (Eakin and Westfall, 1962; Flood, 1975b).

4. Tail musculature

The tail musculature is the most well studied of all the developed organ systems of tunicate tadpoles (*e.g.*, Berrill and Sheldon, 1964; Terakado, 1972; Ohmori and Sasaki, 1977; Whittaker *et al.*, 1977; Meedel and Whittaker, 1979; Whittaker, 1979a), and there are already several excellent ultrastructural reports (Pucci-Minafra, 1965; Castellani *et al.*, 1972; Cavey and Cloney, 1972, 1974, 1976; Bone *et al.*, 1977;

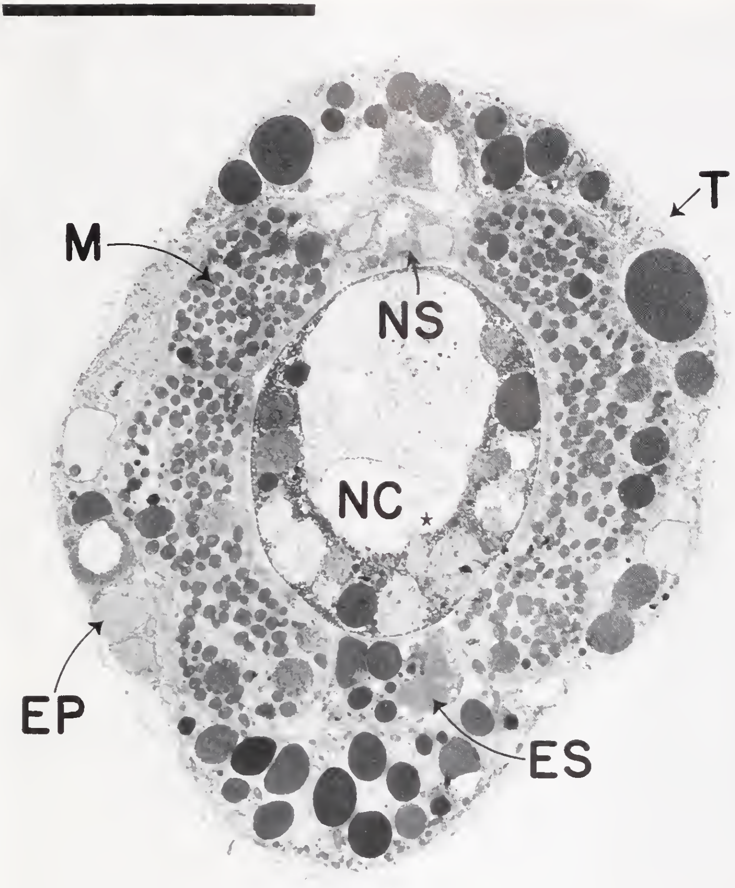

FIGURE 8. Electron micrograph of cross-section of tunicate tadpole tail. Star"*" indicates apparent exocytosis of granular material into notochordal lumen. EP = epidermis, ES = endodermal strand, M = muscle cell, NC = notochord, NS = deuterencephalon of the nervous system, T = tunic. Bar = 10 μm. (Dorsal is at the top.)

Burighel *et al.*, 1977; Cavey, 1980). Therefore, I will only briefly describe the main features of the tail muscle system.

In *Ciona* tadpoles, the tail musculature comprises two sets of striated muscle cells. Each set of muscle cells is aligned as three longitudinal bands flanking the notochord laterally (Figs. 8, 14). Individual muscle cells are fusiform longitudinally and bean-shaped in cross-section. The dorsal and the ventral muscle cells are about 6 × 7.5 μm in cross-sectional diameter, and the middle muscle cells are about 6.5 × 10 μm in cross-sectional diameter. Muscle cells appear to be mononucleated; the nuclei are large (up to 5.5 μm in cross-sectional diameter and 15 μm long), irregularly shaped, centrally located, and contain a number of nucleoli (Fig. 7). The cytoplasm surrounding the nucleus is packed with mitochondria.

The myofibrils are all arranged in a single ring in the peripheral cytoplasm of the cell (Figs. 7, 8, 14). Myofibrils show the general vertebrate banding pattern

(Burighel *et al.*, 1977). Each muscle cell contains 20–35 myofibrils (each is 0.35 × 0.6 μm in cross-sectional diameter), and all the myofibrils are aligned in parallel, slightly oblique to the long axis of the muscle cell, *i.e.*, the long axis of the tail (Pucci-Minafra, 1965). There is no system of T-tubules. (T systems appear to be found only in tadpoles of those tunicate species with more centrally located myofibrils [Bone and Ryan, 1975; Cavey and Cloney, 1976; Burighel *et al.*, 1977]). Sarcoplasmic reticulum tubules completely surround each of the myofibrils. Between each Z band and the adjacent cell membrane there is a flattened and wider tubule or cisterna—these widened cisternae represent probable sites of electro-chemical coupling between cell membrane and myofibril (Burighel *et al.*, 1977).

Neuromuscular transmission is cholinergic (Mendes and Zingales, 1972; Ohmori and Sasaki, 1977; see also: Meedel and Whittaker, 1979), as is apparently the case for all complex metazoans except the arthropods (Mendes *et al.*, 1970; Gerschenfeld, 1973; Krnjevic, 1974; Rovainen, 1979).

5. Adhesive organ

Beginning at metamorphosis, the adhesive organ serves to attach the tunicate to a settlement surface (Cloney, 1977a). An adhesive organ consists of three cone-shaped protrusions—called ‘papillae’—at the anterior end of the tadpole (Figs. 1, 2). The papillae of *Ciona* have a triangular arrangement, with one lying ventrally and two lying dorsally. In the 19–20 h tadpole, each papilla extends about 6 μm from the anterior end of the animal.

The papilla is a mass of packed cuboidal or spherical cells, each about 5 μm in diameter (Fig. 9). The cells are filled with quite dilated RER; they have few yolk vesicles and moderate-sized nuclei (3 μm in diameter). These cells resemble the ‘collocytes’ described by Cloney (1977b) for another suborder of ascidian. Except at their anterior tips, the papillae are enveloped by a single fairly uniform layer of flattened epidermal cells. Cloney (1977b) provides a detailed ultrastructural description of the papillae of a more complex ascidian larva, *Distaplia occidentalis.*

6. Nervous system

From its very beginnings, the central nervous system (CNS) of the tunicate tadpole is unlike that of any invertebrate. Most notably, the tunicate nervous system forms dorsally from a classic chordate neural plate, which then rolls into a neural tube (Kowalevsky, 1866; Conklin, 1905; Satoh, 1978). This neural tube establishes the dorsal tubular CNS of the tadpole.

In the 19–20 h *Ciona* tadpole, the CNS extends most of the length of the animal. Rostrally, it abuts the pharyngeal-gut junction, protruding under the primordial pharynx, *i.e.*, the incurrent siphon rudiment. Caudally, it reaches the end of the tail. The entire CNS is surrounded by a basal lamina averaging 150–250 nm in thickness. At 19–20 h, the CNS lumen is greatly expanded rostrally (to a maximum diameter of about 35 μm), is closed off entirely near the caudal end of the tadpole trunk, and is opened with a small uniform bore (about 1.5 μm in diameter) throughout the tail. By a number of anatomical criteria, the CNS can be divided into two parts: a *prosencephalon* rostrally and a *deuterencephalon* caudally (Fig. 10). (Kuhlenbeck [1975, 1977] provides an extensive review of the vertebrate prosencephalon and deuterencephalon.)

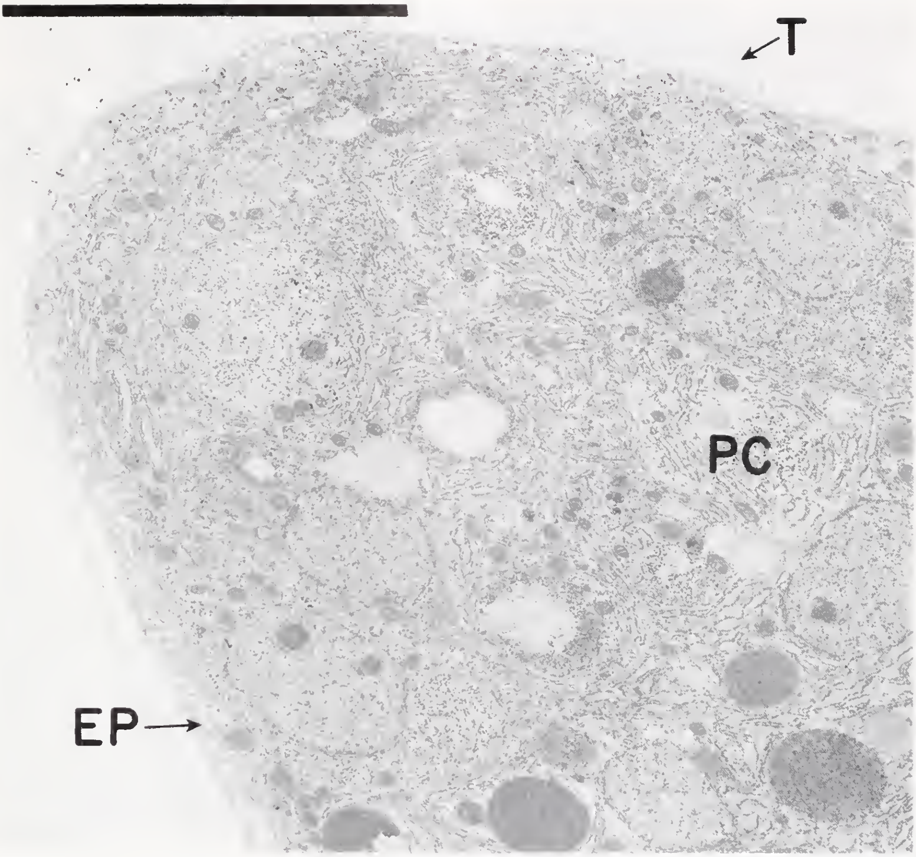

FIGURE 9. Electron micrograph of longitudinal section through one papilla of the adhesive organ. EP = epidermis, PC = papillar cell, T = tunic. Bar = 10 μm.

a. Prosencephalon

The prosencephalon is the bulbous anterior end of the CNS. It is located over the endodermal cavity of the gut primordium. Throughout its length, the prosencephalon tends to be only one cell thick. The prosencephalon contains a single large ventricle, along the inner walls of which lie two pigment cells. Ventrally in the midline, the ventral melanocyte ('VPC' in Figs. 2, 4, 11) sits like a golf ball and tee in the center of the ventricle. The ventral melanocyte has long been thought to be a sensory receptor (Kowalevsky, 1866; Dilly, 1962; Eakin and Kuda, 1971), and it is often called the 'otolith'. Its pigment—melanin (Whittaker, 1966, 1973, 1979b)—is collected intracellularly in a single hemispherical vesicle (with a radius of about 6 μm) capping the cell dorsally. The cell itself is round (13 μm in diameter) with a short ventral neck. The nucleus (4 μm in diameter) has slightly irregular contours, and the cytoplasm contains a number of yolk vesicles. The ventral melanocyte gives rise to an axon that passes caudally along the ventral rim of the prosencephalon and then into the deuterencephalon.

More caudally and dorsolaterally and to the right of the midline, the dorsal melanocyte ('DPG' in Fig. 11) lies embedded in the wall of the prosencephalon. The

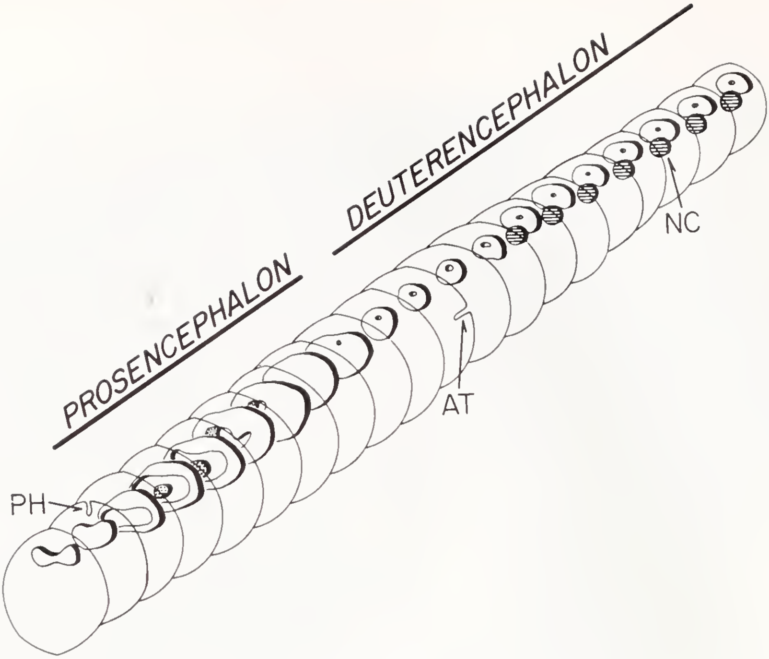

FIGURE 10. Series of sagittal camera lucida tracings through the CNS of the tadpole body (left to right is rostral to caudal). The prosencephalon of the CNS runs from beneath the primordial pharynx to just past the middle of the body. After a brief transitional zone, the deuterencephalon begins (at about the level of the atrial primordia) and runs along the dorsum of the notochord through the tail. The dorsal and the ventral melanocytes can be seen (dotted figures) in the ventricle of the prosencephalon. AT = atrial primordium, PH = primordial pharynx, NC = notochord.

dorsal melanocyte is part of the photoreceptor organ or 'ocellus' (Eakin, 1973). In this pigment cell, the melanin is found in a number of small (0.2–0.5 μm diameter), irregularly shaped vesicles distributed evenly throughout the apical cytoplasm. The dorsal melanocyte is columnar, with a length of 10–12 μm and a width of 3–4 μm in the wall of the prosencephalon. The luminal (apical) end of the cell then extends ventrally another 11–12 μm through the ventricle across the surfaces of the ventrally adjacent photoreceptor cells. The ciliary ends of these photoreceptors protrude 2.5–3.5 μm into the ventricle. The basal end of the pigment cell reaches the basal lamina of the CNS. The cytoplasm of the dorsal melanocyte is slightly paler than the cytoplasm of the adjacent photoreceptor cells, and its nucleus has an irregular outline, with a maximum diameter of about 4 μm.

After traversing the surfaces of the photoreceptor cells, the dorsal melanocyte makes contact with luminal filopodia of the largest cells in the CNS, the lens cells (Fig. 11). The *Ciona* tadpole has three lens cells (Eakin and Kuda, 1971). The lens cells are generally spherical with a diameter of about 18 μm, and they lie side-by-side in the right lateral wall of the prosencephalon. Their peripheral cytoplasm has a dense finely granular texture and contains many mitochondria. These cells contain large (up to 10 μm in diameter) spherical regions of cytoplasm that are free of organelles and that are filled with a finely granular material, more densely concentrated in the center of the regions. These large homogeneous regions are ringed by mitochondria and stain metachromatically with toluidine blue. Detailed ultrastructural descriptions of the dorsal melanocyte, the ciliary photoreceptor cells, and the lens cells of *Ciona intestinalis* tadpoles have been reported by Dilly (1964), Eakin and Kuda (1971), and Eakin (1973). (See also Barnes [1971, 1974] for similar de-

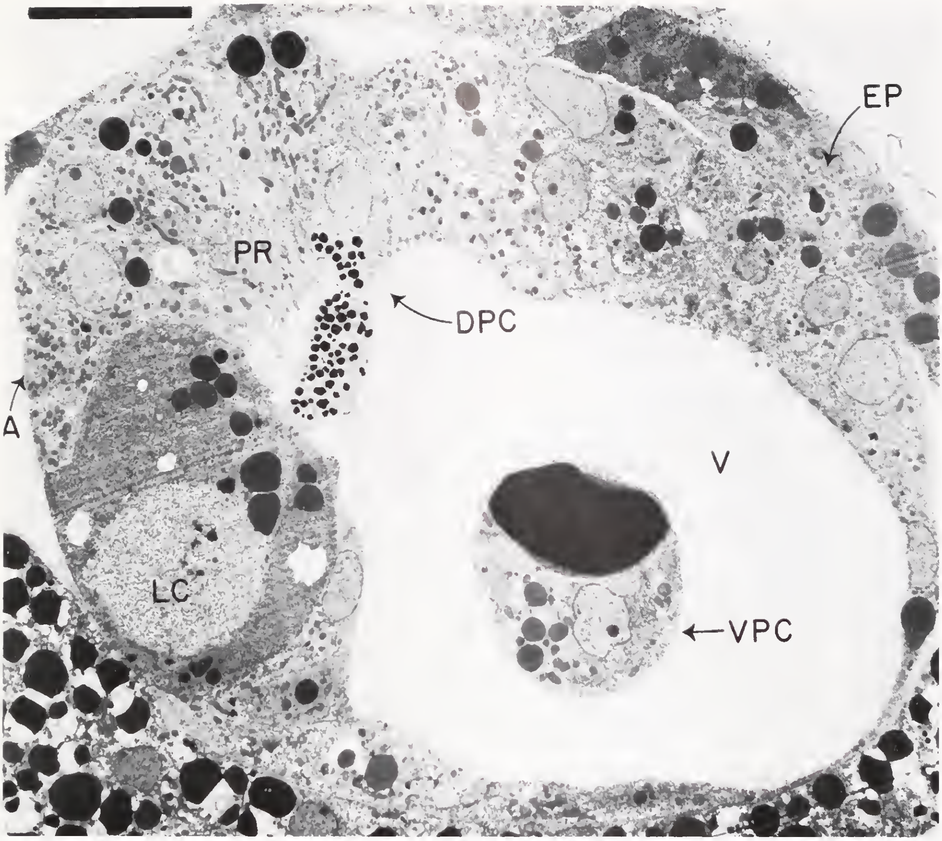

FIGURE 11. Electron micrograph of cross-section through the center of the prosencephalon. A = photoreceptor axons, DPC = dorsal melanocyte, EP = epidermis, LC = lens cell, PR = photoreceptor cells, V = ventricle, VPC = ventral melanocyte. Bar = 10 μm.

scriptions of the tadpole of the tunicate *Amaroucium constellatum*, and see Dilly and Wolken [1973] for a description of the ocellus of adult *Ciona*. Eakin [1973] and Salvini-Plawen and Mayr [1977] provide comprehensive general discussions of the evolution of visual organs.)

Yet another striking cell type characterizes the prosencephalon. A group of generally fusiform cells (4–5 μm wide) in the left ventro-caudal wall of the ventricle protrude tubulated bulbs (1–2 μm in diameter) into the ventricular lumen (Fig. 12). Each bulb sits on a ciliated stalk (9 + 0), and the axoneme runs up one edge of the bulb just inside the cell membrane. The remainder of the bulb is packed with generally parallel arrays of short membranous tubules with a fairly uniform diameter of 0.15–0.25 μm. The cytoplasm of the cell bodies is slightly more granular than the cytoplasm of the surrounding cells, and there also appear to be more mitochondria. Eakin and Kuda (1971), who report that occasional tubules of the bulbs open into the ventricular lumen, provide further ultrastructural descriptions of these unusual cells. A number of investigators (Dilly, 1969b; Eakin and Kuda, 1971; Olsson, 1975) have pointed out the remarkable structural similarities between the tubulated bulb cells of the tunicate CNS and the coronet cells of the teleost CNS. (See Jansen and Flight [1969], Harrach [1970], Galer and Billenstein [1972], and Rossi and Palombi [1976], for descriptions of the coronet cells.)

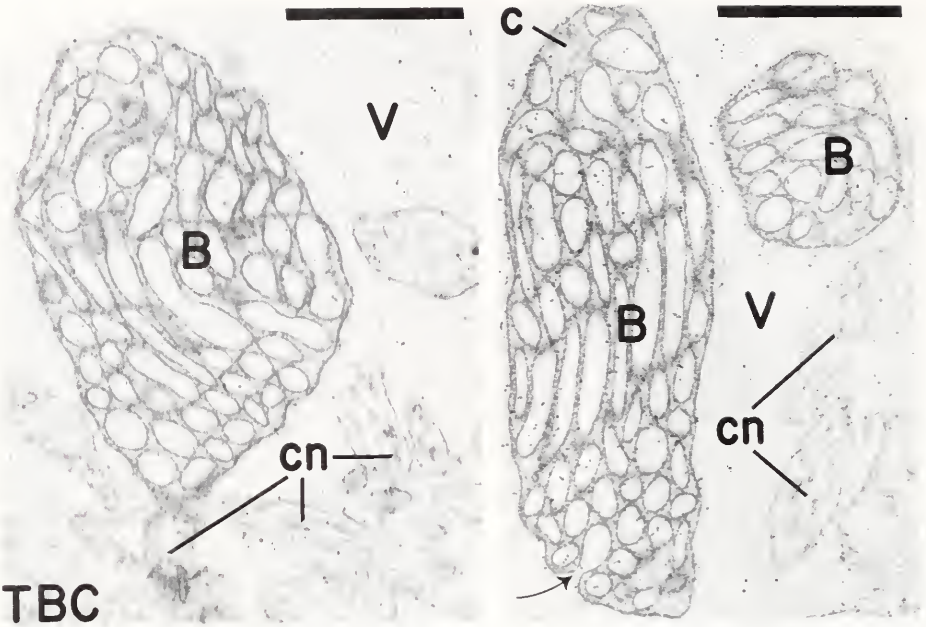

FIGURE 12. Electron micrographs of tubulated bulbs in the ventro-lateral prosencephalon. Arrow points to a natural opening between a membranous tubule of a bulb and the ventricle. B = tubulated bulb, c = "cilium" or axoneme of tubulated bulb, cn = ciliated necks of various tubulated bulb cells, TBC = tubulated bulb cell, V = ventricle of prosencephalon. Bar = 1 μm.

The prosencephalon contains two major axon tracts. On the right side, one axon tract of approximately 20 axons arises dorso-caudally from the bases of the photoreceptor cells and then courses caudally and ventrally as a flattened bundle of axons along the rim of the CNS (Fig. 11). The axons are 0.7–1.2 μm in diameter, and they run just inside the basal lamina, often in grooves of the endfeet of certain CNS cells. On the left side, another major axon tract (also approximately 20 axons) appears to arise ventrally from the vicinity of the tubulated bulb cells. These axons are 0.8–1.5 μm in diameter. They collect ventrally just inside the basal lamina of the CNS and then course caudally as a distinct bundle (still inside the basal lamina) along the ventral rim of the CNS. Both major prosencephalon tracts appear to contribute to the median basal axon tracts that form in the transitional CNS, between the prosencephalon and the deuterencephalon. This impression must still be confirmed by more detailed axon tracing techniques. In the prosencephalon, it is rare for any axons to be completely wrapped by a cell process of a surrounding cell, and there are no myelinated axons anywhere in the CNS of the *Ciona* tadpole. Myelination is a vertebrate characteristic (Peters *et al.*, 1976): truly myelinated axons probably do not exist in invertebrates (Bullock and Horridge, 1965; Bunge, 1970; Guthrie, 1975). Interestingly, lampreys, the most primitive extant vertebrates, also have no myelinated axons (Rovainen, 1979).

b. Deuterencephalon

Caudal to the prosencephalon, the CNS goes through a transitional zone. This region of the 19–20 h tadpole has a dynamic appearance—it is filled with growing cell processes (presumably axons), and it contains much extracellular space. The transitional zone bulges to the right side of the animal behind the photoreceptors and the lens cells, and the deuterencephalon emerges caudally from the left half of the transitional zone.

The deuterencephalon begins in the caudal body at the head of the notochord and extends the length of the tail. The deuterencephalon lies directly along the dorsal surface of the notochord (Fig. 10). Just as with the prosencephalon, the deuterencephalon is a tube that is either one or two cells thick and that is completely surrounded by a fairly uniform basal lamina. Rostrally, the deuterencephalon is greater than 18 μm wide and 25 μm high; caudally, it tapers to less than 4 μm wide and 6 μm high.

A characteristic of the deuterencephalon at almost all levels is that its lumen is lined by four ciliated ependymo-glial cells, and four rows of these nonneuronal cells run in single files along the length of the caudal CNS. These cells appear to each have at least one 9 + 2 cilium, which can be more than 10 μm long.

Dorsally, a capstone ependymo-glial cell constitutes the entire dorsal wall of the deuterencephalon. This cell is wedge-shaped in cross-section and fusiform longitudinally. The capstone cell has "loose" vacuolated cytoplasm. Its nucleus is spherical or oblong (3 μm in cross-sectional diameter) with coarsely granular nucleoplasm.

Laterally, each ependymo-glial cell is crescent shaped in cross-section and fusiform longitudinally. Its nucleus is spherical (3–3.5 μm in diameter), with relatively dark and finely grained nucleoplasm. The ventral half of the basal surfaces of the lateral cells border the dorsal muscle cells. In the rostral deuterencephalon, the ventral surfaces of the lateral ependymo-glial cells border the two ventral deuterencephalic neurons. In the caudal deuterencephalon, axons of the lateral basal tracts run inside the basal lamina in grooves along the ventro-lateral surfaces of the lateral ependymo-glial cells.

Ventrally, the floor of the deuterencephalic ventricle is formed by a cuboidal cell. The floor cell has an oblong nucleus (1.5×4 μm cross-sectionally), and its cytoplasm contains many mitochondria and much dilated RER. In the rostral deuterencephalon, each floor cell lies above the median basal axon tract(s). In the caudal deuterencephalon, each floor cell extends to the ventral basal lamina of the CNS, thereby constituting the entire ventral wall of the deuterencephalon.

In the rostral deuterencephalon, a median basal axon tract (or perhaps a pair of adjacent axon tracts) runs between the ventral surface of the floor cell and the ventral basal lamina of the CNS (Fig. 13). The median basal tract(s) contains approximately 20–25 axons, ranging in diameter from 0.5 μm to 1.0 μm. These axons have descended from the prosencephalon and the transitional zone between the prosencephalon and the deuterencephalon. Some axons of the median basal tract(s) synapse in the rostral deuterencephalon. These synapses appear to include axo-somatic synapses (Fig. 13)—a characteristic of vertebrate nervous systems (Bullock and Horridge, 1965; Cohen, 1970; Bullock, 1974; Gutherie, 1975—Dilly [1975] reports finding axo-somatic synapses in one group of hemichordates but not in another [Dilly *et al.*, 1970]). The median basal tract(s) ends in the rostral tail.

In the caudal deuterencephalon, a lateral basal axon tract runs between the ventrolateral surfaces of the lateral ependymo-glial cell and the basal lamina, on each side of the CNS. Each of these tracts contains approximately 8–10 axons, ranging in diameter from 0.5–1.8 μm (Fig. 14). Axons of the lateral basal tracts make synapses with the dorsal muscle cells in the tail (Fig. 14). (See Flood [1975a], Cavey and Cloney [1976], and Mackie and Bone [1976] for descriptions of nerve-muscle junctions in various tunicates.) In addition to the lateral basal tracts, two axons (0.4–0.5 μm in diameter) run dorsally on each side of the caudal diencephalon in ventral grooves of the epidermal cells *outside* the basal lamina of the CNS. The

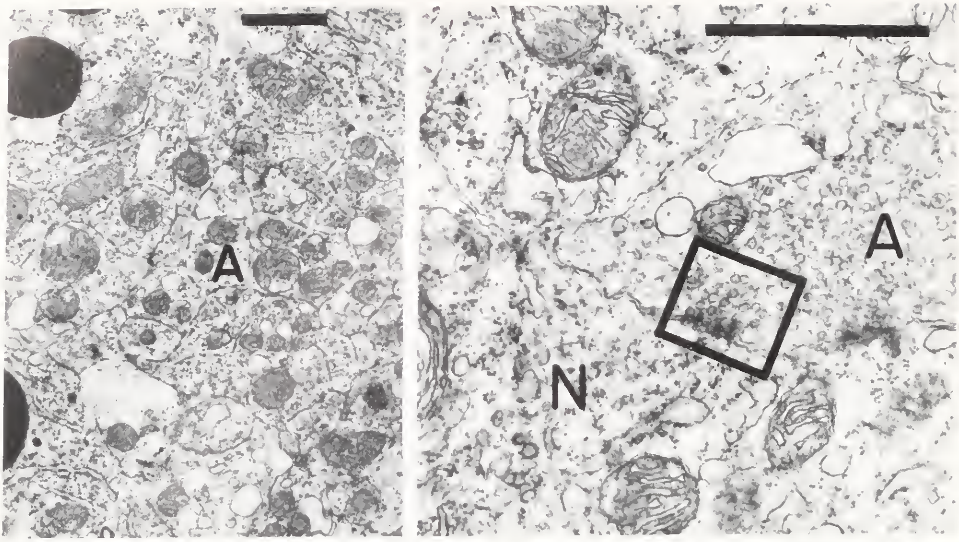

FIGURE 13. Electron micrographs from the rostral deuterencephalon. Left: median basal axon tract(s). Right: axo-somatic synapse (in box). A = axon, N = neuron cell body. Bar = 1 μm.

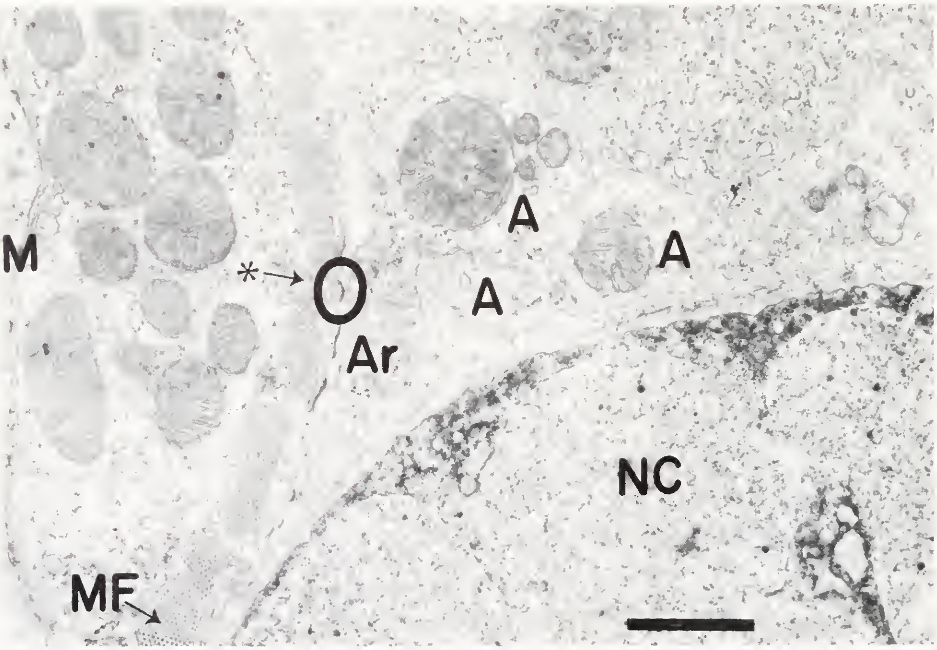

FIGURE 14. Electron micrograph of nerve-muscle junctions in the tadpole tail. Axons are in the lateral basal tract of the deuterencephalon. Asterisk and circle indicate a junction between an axonal rootlet and a dorsal muscle cell; vesicles are accumulated in the pre-synaptic area, and there is a post-synaptic thickening. A = axon, AR = axonal rootlet, M = muscle cell, MF = myofibril, NC = notochord cell. Bar = 1 μm.

exact courses, the origins, and the terminations of these axons as well as further details of the median and the lateral basal tracts must be determined by more specific axon tracing studies. Likewise, specific staining studies must still be done to definitively identify the locations of all CNS neurons.

The general organization of the tail deuterencephalon of *Ciona* tadpoles is quite similar to that of other ascidian tadpoles. Bone and Mackie (1976) found that in *Dendrodoa* a set of ciliated ependymo-glial cells surround the ventricular lumen, and two lateral basal tracts of 5–6 axons each run in grooves along the ventrolateral edges of the CNS. *Dendrodoa* also has a bundle of about 4 axons running through the tail outside the CNS along a groove in the base of the epidermal cells. In contrast to *Ciona, Dendrodoa* tadpoles have a pair of axons running longitudinally (outside the CNS) along the most ventral muscle cells beside the anterior segment of the endodermal strand. Torrence and Cloney (1982) have described a very similar organization in *Diplosoma. Diplosoma* tadpoles do not appear to have a pair of axons running between the ventral muscle cells and the endodermal strand, but they do have a bundle of about 50–70 ventral axons running below the endodermal strand, along a groove in the base of the epidermal cells, in a sort of mirror-image of similar dorsal bundles of axons above the deuterencephalon. These mirror-image dorsal and ventral axon bundles arise from pairs of ciliated sensory cells embedded in the tail epidermis along the midline.

Organ system rudiments

1. Primordial pharynx

A one cell-thick pocket of cuboidal and columnar cells forms the primordial pharynx in the dorsal epidermis of the tadpole body. The primordial pharynx is an oblong tubular body capping the rostral end of the endodermal cavity and then extending caudally over the rostral tip of the CNS (Figs. 2, 4). The external opening of the lumen of the primordial pharynx is directly over the CNS. Ventrally, the ventricle of the CNS prosencephalon extends as a small bore (3–4 μm diameter) ciliated tube under the primordial pharynx. The most rostral end (the 'neuropore') of this ventricle opens into the rostral end of the lumen of the primordial pharynx.

The cells of the primordial pharynx are about 6 μm wide and 9 μm long. Their cytoplasm is slightly darker and has a more finely grained texture than that of the surrounding epidermal cells. The primordial pharyngeal cells are filled with RER in all states of dilation. Nuclei are spherical (3–4 μm in diameter). The luminal surfaces of the primordial pharyngeal cells are fairly smooth and are covered with forming coated pits. In the region of the neuropore, the floor of the primordial pharynx is formed by cells of the prosencephalon. These cells protrude microvilli and cilia into the rostral lumen of the primordial pharynx.

2. Atrial primordia

Pockets of similar cuboidal and columnar cells also form the two atrial primordia, one on the left and one on the right, in the epidermis of the body wall (Figs. 4, 10, 15). The atrial primordia border the gut primordium at a level just rostral to the head of the notochord. Each atrial primordum is a spherical pocket with walls which are one cell thick; at its widest cross-section (28–30 μm diameter), an atrial primordium consists of five or six cells (Figs. 10, 15). Each atrial cell is about 8–10 μm wide and 13–15 μm long. Its cytoplasm has dilated RER. Nuclei are oblong or spherical (3–5 μm in diameter). The luminal surfaces of the cells of the atrial pri-

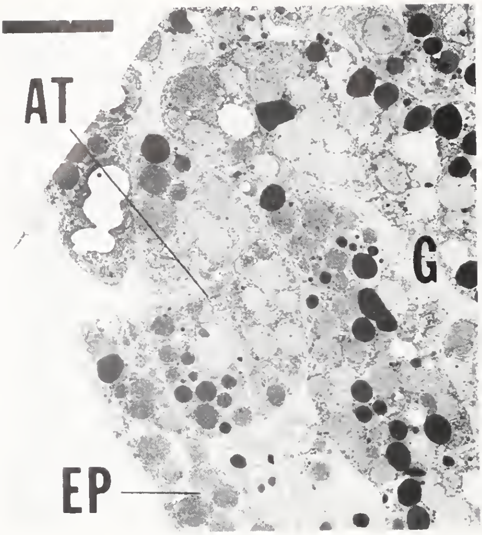

FIGURE 15. Electron micrograph of an atrial primordium in the lateral wall of the tadpole body. AT = atrial primordium, EP = epidermis, G = gut primordium. Bar = 10 μm.

mordia are smooth. During metamorphosis, the two atrial primordia fuse and form the single atrial siphon of the adult (Berrill, 1950). For a general description of the development of the atria, see Willey (1893a).

3. Gut primordium

In the 19–20 h tadpole, the gut primordium is a closed folded tube, one cell thick, that fills the ventral two-thirds of the body (Figs. 2, 4, 15, 16). The gut lumen (the 'endodermal cavity') extends from the pharyngeal primordium rostrally to the head of the notochord caudally. The gut primordium is followed in the tail by the 'endodermal strand'—a lumenless set of gut cells, in single file, lying directly under the notochord along its entire length (Figs. 2, 7, 8).

The prosencephalon lies directly along the top wall of the gut primordium. In the rostral half of the tadpole body, the bottom wall of the gut primordium lies directly along the ventral epidermis. The rostral end of the gut primordium borders an extracellular space behind the adhesive organ. This space is called the 'pre-oral lobe'; it contains separated spherical cells and will apparently continue to fill with cells before metamorphosis (Willey, 1893a).

Cells of the gut primordium and of the endodermal strand are roughly cuboidal (4–8 μm wide) and are characterized by large numbers of yolk vesicles. The cytoplasm of these cells appears "loose" and vacuolated and contains few organelles. At 19–20 h, the luminal surfaces of the gut cells are irregular but have neither cilia nor extensive microvilli. At this developmental stage, there do not appear to be any specialized gland cells.

4. Mesodermal pockets

The 19–20 h *Ciona* tadpole contains a number of pockets of mesodermal cells (Conklin, 1905). Four prominent pockets (two bilateral pairs) are found between the ventrolateral walls of the gut primordium and the ventrolateral epidermis (Figs.

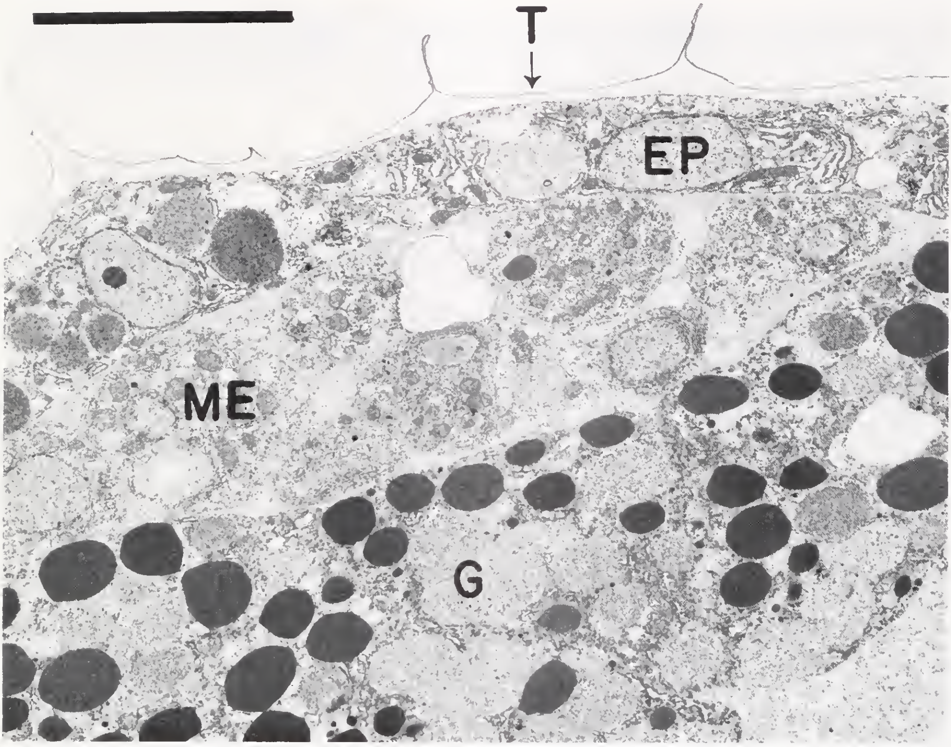

FIGURE 16. Mesodermal pocket in the ventro-lateral body wall of the tadpole. EP = epidermis, G = gut primordium, ME = mesodermal pocket cell, T = tunic. Bar = 10 μm.

2, 4, 16). The mesodermal cells are approximately spherical (5–6 μm in diameter) and are filled with strikingly dilated RER (Fig. 16). The numerous mitochondria appear to be slightly larger (up to 1 μm in cross-sectional diameter) than those of surrounding cells, and the cytoplasm of the mesodermal cells is less granular and contains fewer yolk vesicles than the surrounding cells. Mesodermal cell nuclei are roughly spherical (3 μm in diameter). Presumably, many of these cells will give rise to the musculature of the adult organism.

DISCUSSION

The first important conclusion from these observations is that the gross anatomy in the old descriptions of tunicate tadpoles can for the most part still be accepted today. On the other hand, the fine histological details were often inaccurate in the old reports, and modern ultrastructural analyses must form the bases for anatomical descriptions at the cellular level.

Basically, the tunicate tadpole is an extremely simple organism. In *Ciona* tadpoles, there appear to be fewer than 20 morphologically distinct cell types, which are organized into 6 clearly distinct and well developed organ systems and into about 4 organ system rudiments. Of the developed organ systems, the nervous system—which includes neurons, ependymo-glial cells, pigment cells, and at least two distinct types of receptor cells—contains the greatest variety of cell types. Besides its small number of cell types, the *Ciona* tadpole is also remarkable in the small total numbers of cells that compose its organs. For example, the central nervous system appears to contain fewer than 50 neurons, and this means that the *Ciona* tadpole has what may be the simplest nervous system constructed in the general vertebrate plan.

Vertebrate homologies

Anatomical descriptions of the tunicate tadpole are important to biologists because of the position of the tunicates in chordate phylogeny. It is generally believed that the tunicates of today are descendants of a form of animal that was transitional between the deuterostomian invertebrates (notably, the echinoderms) and the vertebrates (Brooks, 1893; Garstang, 1894, 1928; Van Name, 1921; Berrill, 1955; Romer, 1970; Plough, 1978; Jefferies, 1979). From various biochemical comparisons, Ohno (Atkin and Ohno, 1967; Ohno, 1970; Fisher *et al.*, 1980) has suggested that major gene duplications (either polyploidisations or regional duplications) were instrumental in the divergence of the other chordates from an ancestral tunicate-like stock.

To draw evolutionary inferences from comparative anatomical data, the compared anatomies must be homologous. Homologous anatomies develop from genomic sequences that were identical in an ancestral stock of organisms (De Beer, 1958). Since Kowalevsky first proposed the idea in 1866, many comparative anatomists have considered the body plans of the tunicate tadpole and the vertebrate embryo to be homologues. One strong argument for this homology includes a list of the many sequentially and spatially distinct developmental steps that are apparently shared by the tunicates and the vertebrates. Here, the underlying assumption is that each sequentially or spatially distinct developmental step probably involves at least one different genomic sequence. Thus, many shared sequentially or spatially distinct developmental steps imply a significant number of shared genomic sequences.

As with all deuterostomes (Meglitch, 1972), tunicate embryogenesis begins with a series of fairly straightforward orthogonal cleavages (Conklin, 1905). Later, the blastopore becomes the caudal end of the trunk, and the "mouth" (in the tunicate, the primordial pharynx) develops secondarily at the opposite end of the embryo (Sedgewick, 1889; Willey, 1893a, b; Conklin, 1905). In tunicates, as in all of the vertebrates, the neural tube rolls up dorsally from a dorsal neural plate, the tail develops as a dorsal extension beyond the caudal end of the trunk, the gut develops ventrally, a notochord develops centrally and underlies the entire caudal neural tube, and muscles develop bilaterally flanking the notochord (Kowalevsky, 1866; Willey, 1893a, b; Conklin, 1905; MacBride, 1914; Satoh, 1978). These developmental steps give rise to an organism with a basic vertebrate topology, as diagrammed in Fig. 17. It is intriguing that incomplete gastrulation can lead to a larval vertebrate with a body plan that is even more strikingly like a tunicate tadpole—see figures 6 and 7 in Moore (1946). (In addition, the tunicate endostyle may be homologous to the vertebrate thyroid gland [Brooks, 1893; Barrington, 1957; Thorpe and Thorndyke, 1975].)

Vertebrate embryos typically develop two additional tubular systems (Goodrich, 1930; Neal and Rand, 1936; Ballard, 1964; Poole and Steinberg, 1981). Centrally, between the notochord and the gut, a major artery (or a pair of major arteries) extends the length of the caudal half of the animal. Furthermore, bilateral excretory ducts of the genito-urinary system run adjacent to the arteries through the vertebrate trunk. *Ciona* tadpoles do not appear to develop distinct homologues to these basic

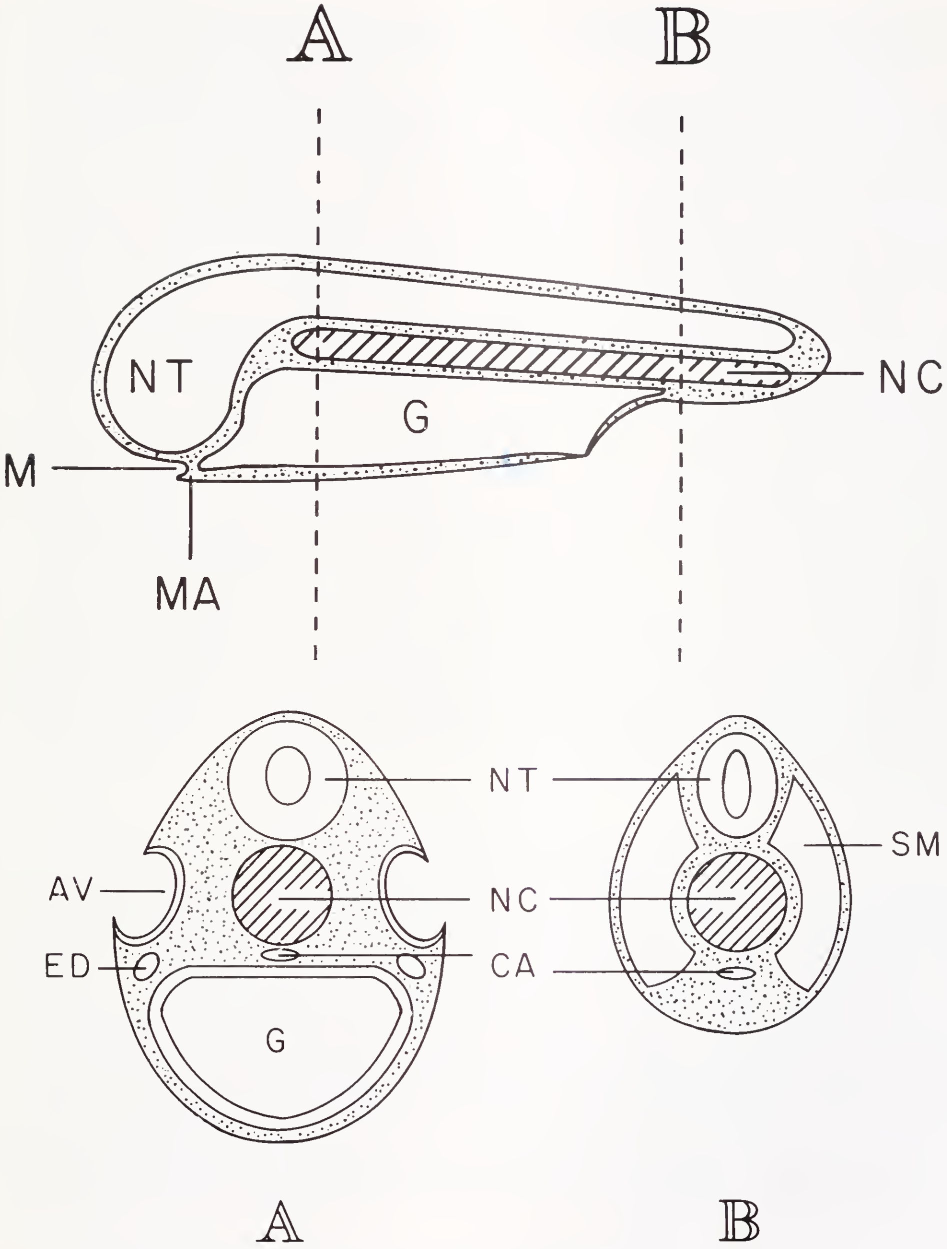

FIGURE 17. a: Schematic sagittal section of a characteristic vertebrate embryo. b: Schematic cross-sections at the two levels indicated in figure 17a. AV = auditory vesicle, CA = central artery, ED = genito-urinary excretory duct, G = gut primordium, M = mouth, MA = mandibular arch, NC = notochord, NT = neural tube, SM = somatic musculature.

vertebrate tissues. On the other hand, these tissues are largely mesodermal in origin, and the bilateral pockets of mesoderm in the tunicate tadpole may be related to the precursor tissues of the vertebrate circulatory and genito-urinary systems. (The tunicate heart does develop from a ventral pocket of mesoderm cells, but in the 19-20 h *Ciona* tadpole this organ has not yet formed [Selys-Longchamps, 1900].)

If the tunicate tadpole and the vertebrate embryo do in fact have essentially homologous topologies, then two other anatomical homologues can be suggested. First, all vertebrates develop auditory vesicles from bilateral invaginations of the body ectoderm at about the level of the head of the notochord (McEwen, 1931; Yntema, 1055; Ballard, 1964; Thornhill, 1972; Jarvik, 1980; Model *et al.*, 1981). The extinct mitrates, probable precursor organisms to all the chordates, also appear to have had homologous bilateral vesicles, called 'atria' (Jefferies and Lewis, 1978; Jefferies, 1981). Moreover, Bone and Ryan (1978) have shown that the atrium of the adult tunicate develops ciliated sensory cells in cupular organs resembling those of the typical vertebrate acoustico-lateralis system. Thus, the bilateral atrial primordia of the tunicate tadpole may be homologous to the inner ear primordia of the vertebrates. (Bone and Ryan [1978] have suggested essentially the same homology.)

Second, vertebrate embryos develop bilateral bulges, the embryonic mandibular arches, just ventral to the mouth (McEwen, 1931; see Neal and Rand, 1936, Bjerring, 1977, and Jarvik, 1980, for discussions of the terminology and the anatomy of the embryonic mandibular arch region, which appears to develop two distinct somites—a premandibular somite and a mandibular somite—in many vertebrates). In amphibians, transient adhesive organs develop at the ends of these bulges (Harrison, 1925; McEwen, 1931; Schotte and Edds, 1940; Eakin, 1963; Perry and Waddington, 1966; Lyeral and Pelizzari, 1973; Roberts and Blight, 1975). There may also be homologous adhesive organs in the larvae of teleost fishes (Kerr, 1919). (But see Garstang [1928] for an alternative discussion.) Might the adhesive organs and their foundation tissues in the tunicate tadpole be homologous with the embryonic mandibular arches of the vertebrates?

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ANTIBACTERIAL ACTIVITY OF THE COELOMIC FLUID OF THE POLYCHAETE, *GLYCERA DIBRANCHIATA*.

I. THE KINETICS OF THE BACTERICIDAL REACTION

BENJAMIN M. CHAIN AND ROBERT S. ANDERSON

Sloan-Kettering Institute for Cancer Research, Donald S. Walker Laboratory,
145 Boston Post Road, Rye, New York 10580

ABSTRACT

The kinetics of the bactericidal action of cell-free coelomic fluid from *Glycera dibranchiata* on a Gram-negative bacterium, *Serratia marcescens*, were studied using both a turbidometric growth assay and a direct plating assay. The bactericidal reaction consisted of two stages involving an initial rapid binding to the bacterial surface, followed by a slower killing reaction. The first stage required the presence of divalent cations and was slightly temperature-dependent. In contrast, the rate of the second stage was accelerated at temperatures normally optimal for multiplication of the bacteria. Kinetic studies and an analysis of the dose-response curve at low concentrations indicated that three or four hits by the bactericidal factor are required to kill a bacterium.

INTRODUCTION

The coelomic fluid of the marine worm, *Glycera dibranchiata*, has been shown to possess potent bactericidal properties against several Gram-negative bacteria. Preliminary studies showed that the bactericidal activity resides in a heat-labile protein with a molecular weight of >100,000 (Anderson and Chain, 1982). *Glycera* antibacterial factor (GAF) presumably acts as part of the natural humoral immunity system of the worm and defines a new class of animal-derived antibacterial molecules. Two major questions of interest are the mode of action and biochemical structure of GAF. This paper will describe kinetic experiments which are aimed at elucidating the first of these questions, the accompanying paper will address the second. Ideally, it would have been more satisfactory to purify and characterize GAF before undertaking kinetic studies, but this proved technically difficult.

The results of these kinetic studies suggest a two-step model for GAF bactericidal action involving an initial binding to the bacterial surface and a subsequent "killing reaction."

MATERIALS AND METHODS

Glycera dibranchiata (blood worms) were obtained from the Maine Bait Co. and kept in artificial sea water aquaria at 12°C. Methods for bleeding the worms and preparing cell-free sterile coelomic fluid (CF) were described previously (Anderson and Chain, 1982).

Two types of assay of bactericidal activity, using overnight cultures of *Serratia marcescens* as the test organism, were adapted for use with *Glycera* coelomic fluid. The first was based on the turbidometric assay of Muschel and Treffers (1956a) for

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measuring complement activity. A known volume of coelomic fluid was diluted in artificial sea water (ASW) to give a total volume of 0.5 ml. GAF activity was greater in ASW than in the phosphate buffer system employed in earlier studies (Anderson and Chain, 1982). A bacterial suspension of *S. marcescens* (~6 × 10^5 bacteria/0.1 ml) was added to the coelomic fluid. After gentle mixing, the bacteria-coelomic fluid mixture was incubated, routinely at room temperature, for a period of 1–30 minutes. The effects of various incubation temperatures were also studied. The incubation was terminated by dilution with 4 ml cold tryptic soy broth (TSB) and the cultures maintained at 1–4°C until all portions of the experiment were complete. The bacteria were then grown up at 37°C until control tubes (without coelomic fluid) reached $O.D._{570} \cong 0.25$. The relative optical densities were a measure of the number of bacteria surviving exposure to coelomic fluid. For quantitative studies, a standard curve was used to convert the relative O.D.s to the number of bacteria surviving exposure to GAF, present at the start of the growth period (Anderson and Chain, 1982).

The other assay used was a direct plating assay. Coelomic fluid and bacteria were allowed to react at room temperature, or at 37°C, for periods of 10 minutes to 2 hours. The suspension was diluted (1:100 in 0.9% saline) to stop the reaction, mixed thoroughly, and 100 μl aliquots were plated in 10 ml tryptic soy agar. The plate counts obtained were a direct measure of the number of bacteria surviving exposure to the coelomic fluid.

Artificial sea water was made up as a 38 g/l solution of Instant Ocean (Aquarium Systems, Inc.). The concentrations of the major ionic components were Na^+, 443 mM, K^+, 9.5 mM, Ca^{2+}, 9.4 mM, Mg^{2+}, 50 mM, Cl^-, 518 mM, and SO_4^{2-} 26 mM. All other chemicals were obtained from the Sigma Chemical Company. Dialysis experiments were done at 1°C using Spectrapor 2 membrane tubing (M. W. cutoff, 12,000–14,000).

RESULTS

The nature of the assay

In our initial studies there was an apparent lack of agreement between the data obtained from the two assays described above; these discrepancies are shown in Figure 1. In the turbidometric assay, low concentrations of CF produced a stimulatory effect on bacterial growth not seen in the plate assay. This was probably due to the stimulatory effect of certain metal ions present in CF on the subsequent growth of the bacteria. When the reaction was carried out in ASW rather than in sodium phosphate buffer (0.1 M, pH 7.5), no stimulatory effect was observed (Fig. 2). In addition, the time required to run the assay was considerably shortened. The action of divalent cations in shortening the lag phase of bacterial growth has been well documented (*e.g.*, Guirard and Snell, 1962).

The total bactericidal activity of CF was also greatly enhanced in ASW (Fig. 2). The CF volume required for a 50% inhibition of optical density was reduced from 18.6 ± 1.9 μl ($\bar{X}$ ± SEM, N = 5) to 4.6 ± 0.5 μl ($\bar{X}$ ± SEM, N = 11). The ionic requirements of GAF were studied further in dialysis experiments. Coelomic fluid was dialyzed overnight against a large volume of Tris buffer (0.1 *M*, pH 8) containing 0.01 *M* $CaCl_2$ shown to be required to prevent irreversible loss of activity. The dialyzed CF was assayed for bactericidal action in media of different composition, and the activity (the volume of CF required to obtain a 50% reduction in optical density, relative to controls) was determined (Table I). Nearly total original activity of dialyzed CF was restored when tested in ASW rather than in dialysis buffer.

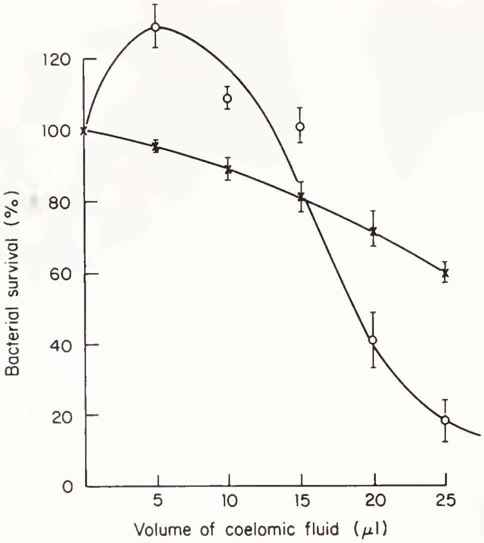

FIGURE 1. A comparison of the turbidometric assays (O) and plate assays (X) of bactericidal activity; mean ± S.E.M. (N = 3). Incubations were for 30 min at room temperature in sodium phosphate buffer (pH 7.5, 0.1 *M*).

Maximal activity was observed when magnesium and sodium ions in concentrations approximating those found in sea water, were added to the dialysis buffer.

Another major discrepancy between the two assays was the much greater apparent activity recorded by the turbidometric assay, in comparison to the plate assay (Fig. 1). In the hope of clarifying this anomaly, the changes in bacterial numbers

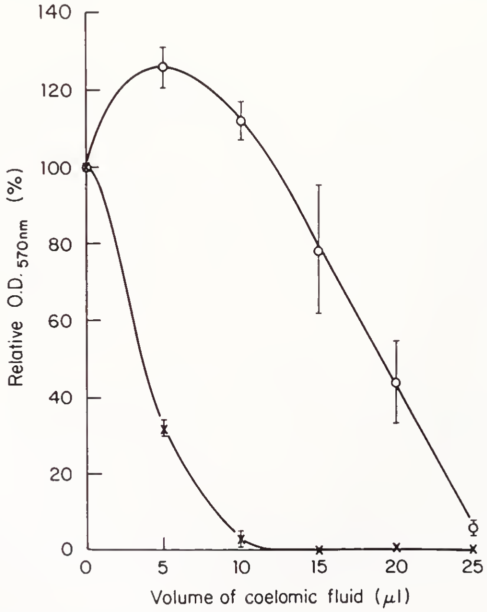

FIGURE 2. A comparison of the turbidometric bactericidal assay in sodium phosphate buffer (O) and in artificial sea water (X); mean ± S.D. (N = 3). Incubations were for 30 min at room temperature.

TABLE I

The influence of assay medium on bactericidal activity

Treatment of CF	Assay medium	CF Volume (μl) required for 50% reduction in O.D.
Undialyzed	Artificial sea water	4.8
Dialyzed	Artificial sea water	5.5
Dialyzed	DB (Dialysis buffer: 0.01 *M* Tris, pH 8, containing 0.01 *M* $CaCl_2$)	14.5
Dialyzed	0.5 *M* $MgCl_2$ in DB	12.0
Dialyzed	0.5 *M* NaCl in DB	11.5
Dialzyed	0.1 *M* NaCl inDB	17.0
Dialyzed	0.5 *M* $MgCl_2$ + 0.5 *M* NaCl in DB	5.5

Coelomic fluid was dialyzed overnight against a 0.01 *M* $CaCl_2$ + 0.01 *M* Tris buffer (pH 8.0). Dialysates (0–20 μl) were assayed (in triplicate) using the turbidometric assay and 50% activity values calculated from the dose-response curves obtained.

during the early part of the amplification (bacterial growth) period of the turbidometric assay (*i.e.*, from the time the broth was added to the bacteria until an optical density could be recorded) were measured directly by plating successive samples (Fig. 3). The results were unexpected. The numbers of viable bacteria were almost unchanged throughout the "reaction period" at room temperature and subsequently, for as long as the tubes were maintained at 1–4°C. However, when placed at 37°C, required to start the amplification phase of the assay, bacterial killing occurred immediately. This decline in numbers was followed by a logarithmic increase as the surviving bacteria multiplied. Both the extent of the initial decrease in numbers and the interval before logarithmic growth were directly related to the amount of CF added to the bacteria. The conclusions from these experiments were threefold: 1) as much as 25 μl of CF exhibits little or no killing activity at room temperature or below during the initial incubation. 2) This volume of CF rapidly kills most bacteria during incubation at 37°C. 3) Once CF has been allowed to interact with bacteria in these concentrations at room temperature, without lethal effect, it retains its ability to kill the bacteria when placed at 37°C, even though the reaction mixture has been diluted ninefold with TSB. This killing is not due simply to low levels of CF present after dilution with TSB because 25 μl CF are inactive if added to bacteria already diluted with TSB. Therefore, we must conclude that the CF and bacteria interact or bind during the initial incubation period at room temperature. We showed that this binding could be disrupted by the vigorous mixing in a very diluted medium which occurred during the course of the plating assay so that a spuriously low apparent activity was recorded. When TSB was added to the reaction mixture, GAF remained bound to the bacterium and was cidal at the optimal temperature.

The fact that bacteria/GAF interaction at room temperature could be reversed by vigorous agitation and dilution suggested that the active factor might be bound to the outer surface of the bacterium. We showed that free GAF could be readily inactivated by trypsin (Table II). In view of this, it was of interest to see whether we could demonstrate cell-bound GAF by its trypsin sensitivity. Indeed, the addition of trypsin to the putative bacteria-GAF complex progressively reduced bactericidal

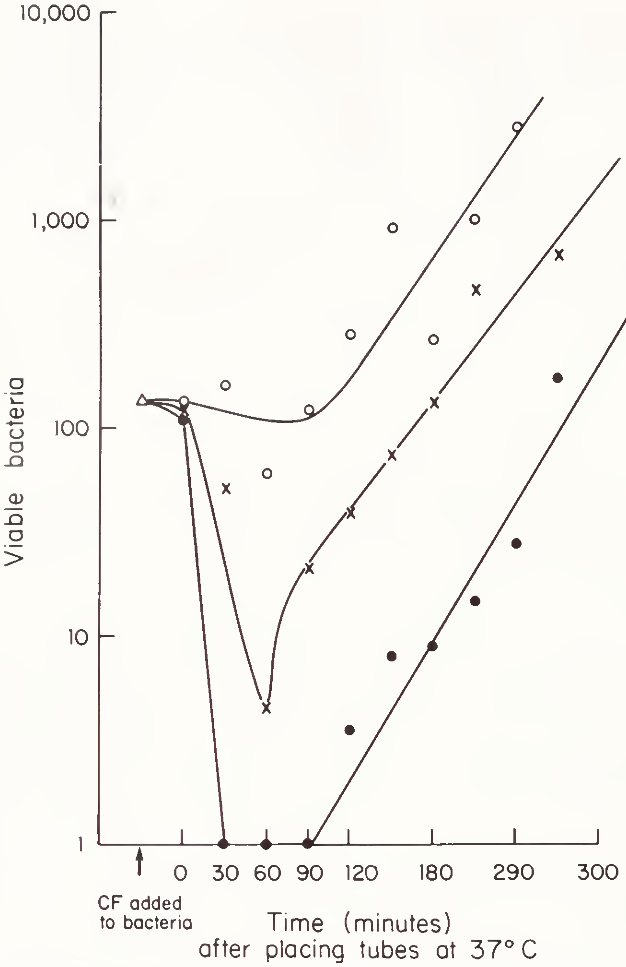

FIGURE 3. Analysis of turbidometric assay using plate counts. The bacteria and coelomic fluid (X = 10 μl CF; ● = 25 μl CF; ○ = no CF) were incubated together at room temperature for 30 min and 4 ml of tryptic soy broth was added to the mixture. A sample was taken from each tube, diluted (1:100) and 100 μl plated. All tubes were immediately placed at 37°C and sampled every 30 min and the number of viable bacteria determined. Points represent the average of duplicate plate counts; to obtain actual number of bacteria, multiply by 4.6×10^3.

activity. These results reinforce the idea that the factor binds initially to the outer surface of the bacterium.

Kinetics of the binding and killing reaction

Killing, as measured by the turbidometric assay, was shown above to consist of two parts: an initial incubation period during which bacteria and GAF associate in a way which can be reversed under certain conditions, and a subsequent period at 37°C (the amplification stage of the assay), during which the bacteria undergo GAF-mediated killing. Assuming that at short incubation periods the limiting factor in the killing reaction is the speed at which "killing units" of GAF attach to bacteria, the turbidometric assay gives a measure of the rate of binding. The kinetics of cell binding by GAF were extremely fast (Fig. 4a). The initial rate of reaction varies very

TABLE II

The trypsin sensitivity of GAF before and after it has bound to the bacterial surface

Treatment (length of incubation)	Effect of trypsin on bactericidal activity of GAF (% bacteria killed)	
	free GAF	bound GAF
No trypsin	100	100
Trypsin (15 min)	15	90
Trypsin (30 min)	0	64
Trypsin (60 min)	0	46

Bactericidal activity was measured by the turbidometric assay using 10 μl CF and a 15 min incubation period. Trypsin, at a final concentration of 200 μg/ml, was added before or after the bacteria/CF incubation as specified above. All trypsin incubations were carried out at room temperature. The presence of trypsin alone was shown not to affect bacterial growth.

sharply with concentration and cannot be accurately measured by this technique at CF concentrations >15 μl. A double logarithmic plot of rate versus concentration (Fig. 4b) has a slope between 3 and 4.

The initial rate of binding as a function of temperature is shown in Figure 5a; over the range 0–37°C an Arhenius plot of the rates (Fig. 5b) lies close to a straight line, with a low Q_{10} of about 1.6.

In contrast to the turbidometric assay, the results of the plate assay are a direct measure of the rate of the first irreversible step of the sequence of events leading to the death of the bacterium. At room temperature the rate of killing is rather slow and extremely variable. Therefore, the relation between rate of killing and concentration of GAF was investigated at 37°C (Figs. 6a, b). Unlike the rate of binding, the rate of reaction approaches a limiting value with increasing concentration. Figure 6c shows that the data, at least at concentrations greater than 5 μl, can be fitted by a hyperbolic function, with a V_{max} of approximately 5×10^5 bacteria/minute. The interpretation of these data will be discussed below, but the comparison of the kinetics of the initial binding to those of the killing reaction are striking. With 10 μl of CF the initial rate of binding (Fig. 4b) is 19.8×10^4 bacteria/minute, while the initial killing rate is 4.3×10^4 bacteria/minute (Fig. 6b). For 15 μl the corresponding figures are 105×10^4 and 6.8×10^4 bacteria/minute. It is apparent that for all but very low concentrations of CF, the bacteria bind GAF before there is any appreciable killing even at 37°C.

The temperature dependence of the killing reaction was difficult to measure accurately because of the variability of the data below 37°C (Table III). However, if one assumes a constant Q_{10} in the range 21–37°C, the data of Table III yield a value of Q_{10} of 2.5 ± 0.4 ($\bar{X} \pm$ S.D.), which is within the range of that found for many biochemical processes.

As has been explained above, since the killing reaction is allowed to go to completion at 37°C, the results of turbidometric assay are a measure of the total bactericidal capacity of a given volume of CF, provided sufficient time is given before the addition of tryptic soy broth for all available GAF to react with bacteria (greater than 15 minutes as shown in Fig. 4). Figure 7a shows the dose response obtained in this way at very low CF concentrations. The curve is sigmoidal, indicating either that the cooperative effect of more than one component of CF is required for bactericidal action, or that a multiple binding of one component is required to kill a bacterium. For multiple hit kinetics, the assumption of Poissonian probability distribution can be used to calculate theoretical dose-response curves (Mayer, 1961;

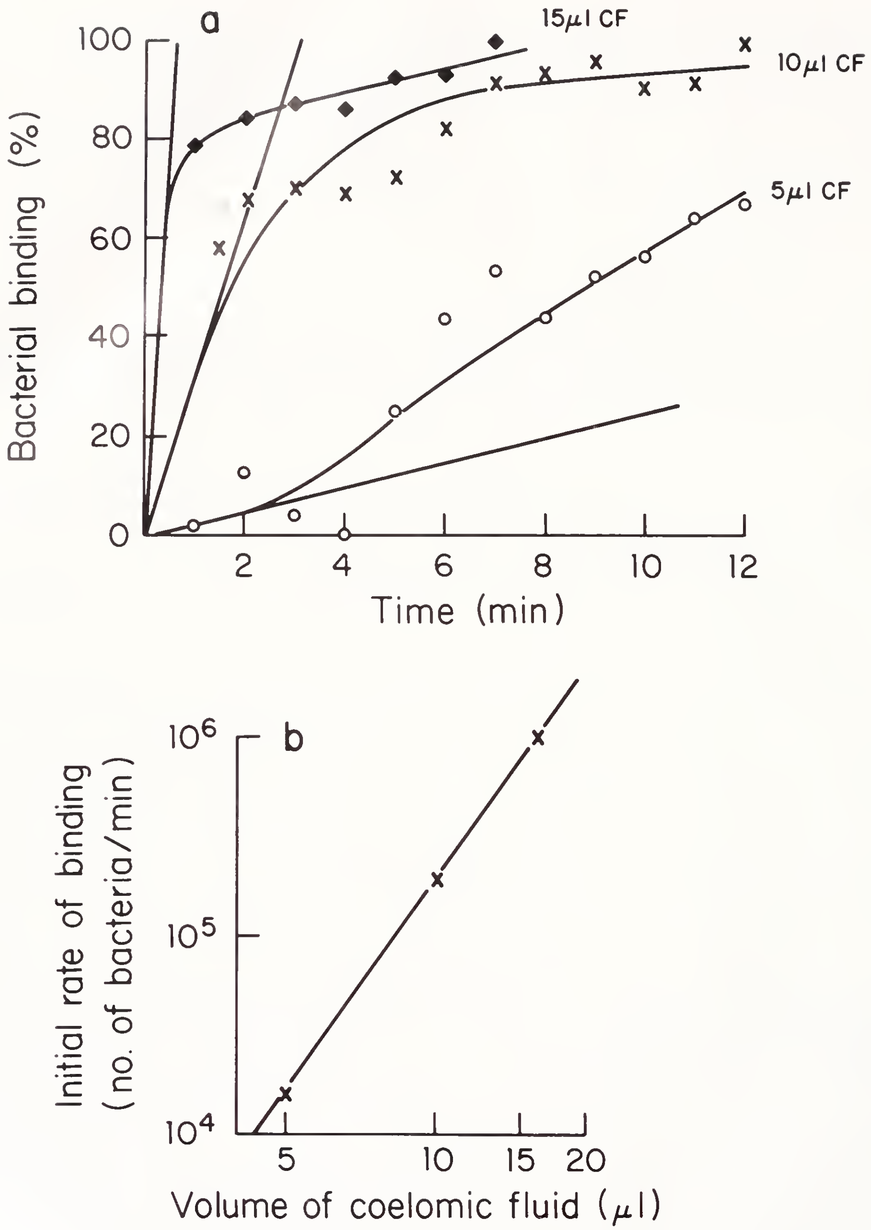

FIGURE 4. a) Rate of binding of GAF to bacteria using different CF volumes (O = 5 µl CF; X = 10 µl CF; ♦ = 15 µl CF). Bacteria were added to a given volume of coelomic fluid and allowed to react for 1–12 minutes. At the end of this period unbound coelomic fluid was diluted out with 4 ml tryptic soy broth and the bacteria grown up in the usual way. All bacteria which had bound sufficient GAF during the original incubation period were killed during the subsequent growth period. Bacterial killing was calculated directly from relative optical density as described in Methods.

b) Initial rates of binding (number of bacteria per min) were calculated from tangents drawn through the origin in Figure 4a. Initial rates are plotted as a function of concentration on a double-logarithmic plot. The slope of the straight line drawn through the three points is 3.8.

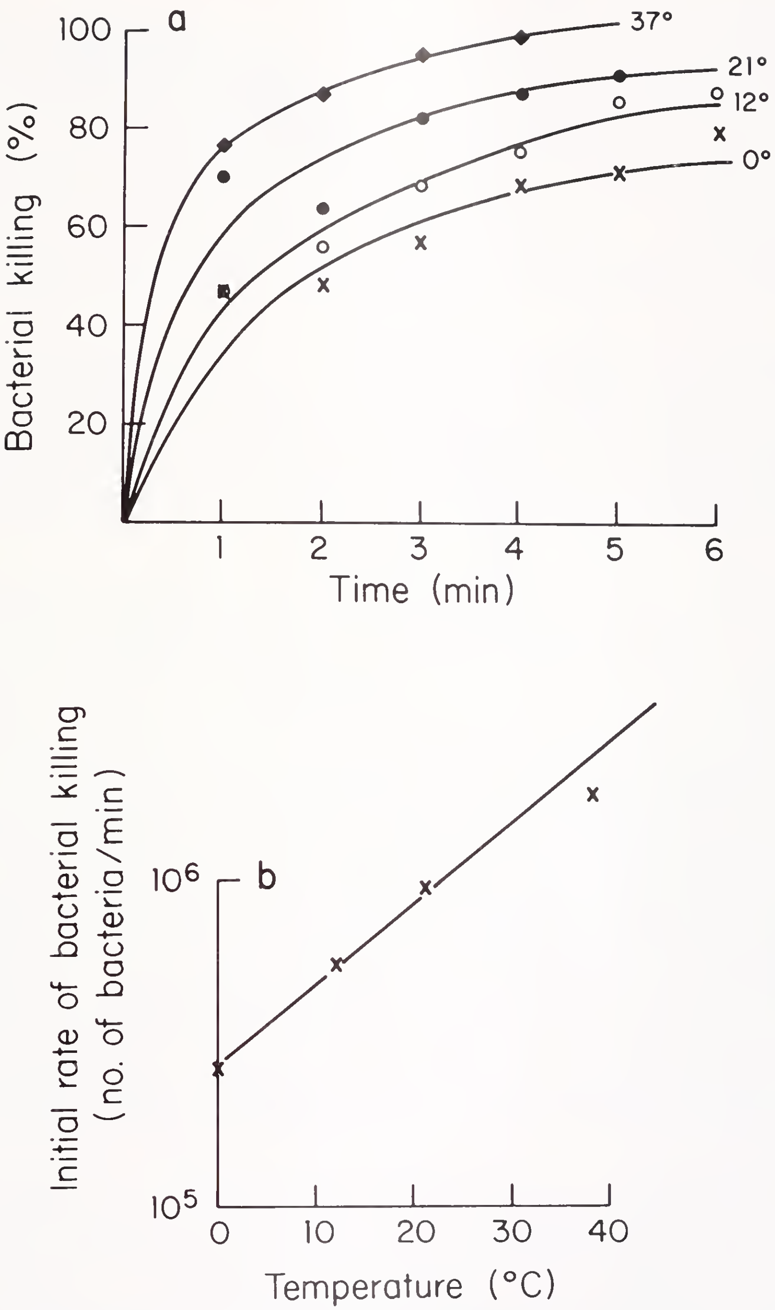

FIGURE 5. a) The rate of binding of GAF to bacteria at different temperatures (X = 0°, ○ = 12°, ● = 21°, ♦ = 37°C). The experiment was carried out as described in Figure 4; 10 μl of coelomic fluid was used in all cases. All determinations were done in triplicate and the average optical density was used to calculate bacterial killing.

b) An Arhenius plot of initial rate of bacterial killing as a function of temperature. The rates of killing were measured from the data shown in 5a, as described in Figure 4b.

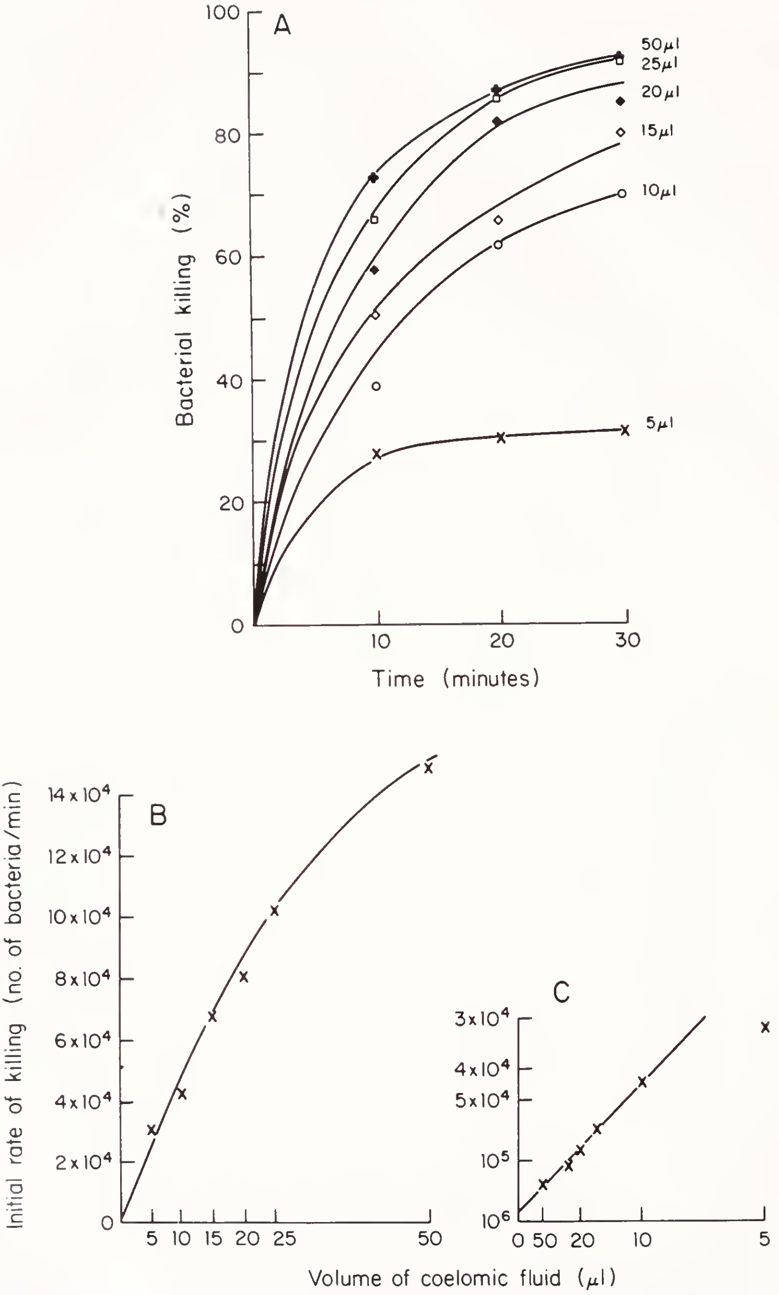

FIGURE 6. a) The rate of bacterial killing with different volumes of coelomic fluid. Bacteria and coelomic fluid were incubated together for the specified time and surviving organisms were counted by plating in tryptic soy agar as described in Methods. All values are the average of triplicate determinations.

b) Initial rate of killing as a function of coelomic fluid volume. The initial rates were obtained from the tangents (at 5 = 0) to the curves in 6a).

c) The data of 6b replotted in a double reciprocal form. The data approximate to a straight line at coelomic fluid concentrations greater than 5 μl. The intercept on the y axis, corresponding to the limiting velocity at high concentrations of coelomic fluid, is 5 × 10^5 bacteria/minute.

TABLE III

Temperature dependence of GAF activity

Temperature	Average initial rate of bacterial killing (bacteria/min)	Standard deviation (N)	Range
21°C	1.2×10^4	0.7×10^4 (4)	$0.6–2.6 \times 10^4$
37°C	5.1×10^4	0.9×10^4 (3)	$4.0–6.3 \times 10^4$

The initial rate of bacterial killing, as measured by the plate assay, was calculated as described for Figure 6.

Wright and Levine, 1981b). Such curves, plotted as the log of the proportion of surviving bacteria versus number of killing units/bacterium present for 1, 2, 3 or 4 hit models, are shown in Figure 7b. The curves in all cases approach a straight line at sufficiently high concentrations, and the intercept on the y-axis gives a measure of the multiplicity. A similar plot for the data in Figure 7a is shown in Figure 7c, and would indicate that 3 or 4 hits are required for the lethal action of GAF.

DISCUSSION

The principal conclusion of this paper is that the bactericidal action of GAF can be broken down experimentally into a two-step process of the type:

$$\text{n(GAF)} + \text{Bacterium} \xrightarrow{k_1} \text{Bacterium-GAF}_n \text{ complex} \xrightarrow{k_2} \text{Bacterium (killed)}$$

The evidence for this two-step model, involving an initial rapid, reversible binding reaction, followed by a slower killing reaction, and the methodology used to study the two processes have been presented in detail in the Results section.

The rate of binding of GAF to the outer surface of the bacterium is extremely rapid. The Q_{10} of this reaction (1.7) also indicates a physical binding type reaction, with a low activation energy, rather than a process involving metabolic reactions. Furthermore, GAF remains trypsin-sensitive when bound to the bacterium, indicating that at least some portion of the molecule is still exposed to the medium. The binding of GAF is, however, strongly influenced by the ionic composition of the medium. Not surprisingly, optimum binding occurs in ASW whose composition is very similar to *Glycera* CF.

Two separate pieces of evidence suggest that more than one molecule of bound GAF is required to kill a bacterium. From the law of mass action, the initial rate of binding ($V_{t=0}$) should be given by:

$$V_{t=0} = R_1(\text{GAF})^n[\text{bacteria}]$$

If the number of bacteria is kept constant, this can be rewritten as:

$$\text{Log } V_{t=0} = n \log [\text{GAF}] + \text{constant}$$

where n, the slope of this logarithmic relationship, is the number of molecules required for a lethal hit. A double-logarithmic plot of initial rate data was shown in Figure 4b and has a slope of 3.8, suggesting that between 3 and 4 molecules of GAF are required to kill a single bacterium.

An alternative approach to test the multiple-hit model is to allow the binding of free GAF to go to completion and look at the dose-response curve at low GAF levels (Fig. 7). As has been pointed out above, the theoretical dose-response curves

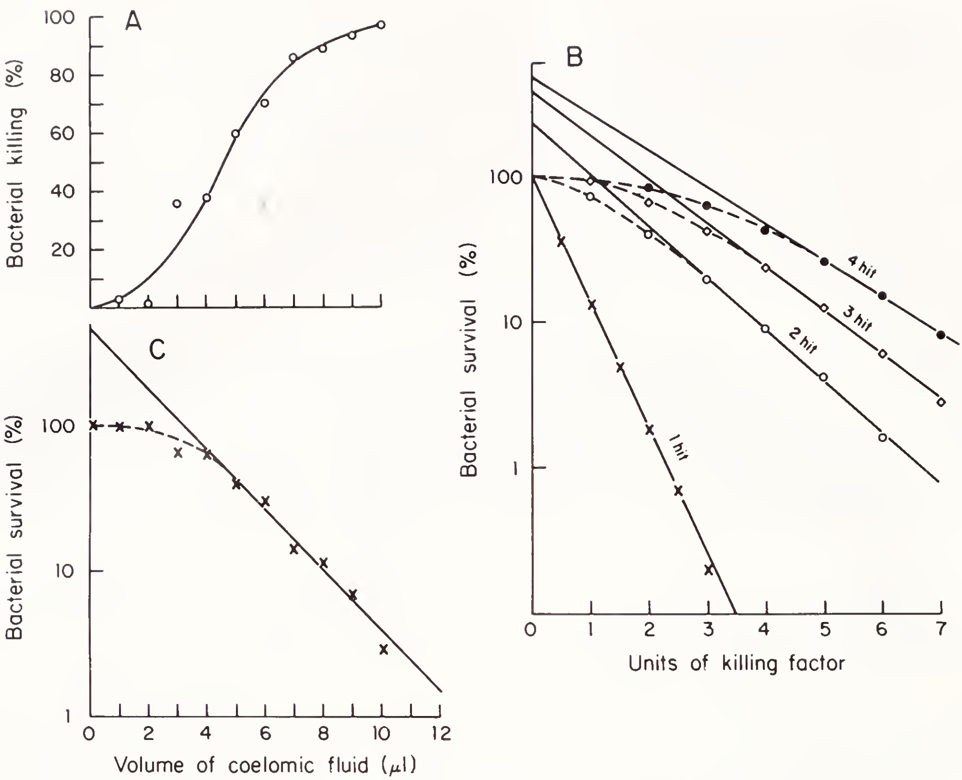

FIGURE 7. a) Dose-response curve at low volumes of coelomic fluid using the turbidometric assay. Bacteria and coelomic fluid were incubated together at room temperature for 30 min to allow complete binding of all free GAF before the addition of tryptic soy broth. Each point is the average of 5 determinations.

b) Theoretical curves of log (% survival) as a function of units of killing factor per bacterium for 1, 2, 3 and 4 hit models. Values are calculated from the Poisson distribution:

$$P_n = \left(1 + \lambda + \frac{\lambda^2}{2!} \cdots + \frac{\lambda^n}{n!}\right) e^{-\lambda}$$

where p is the probability of a bacterium binding n units of killing factor and λ is the total number of units present for each bacterium. $P_n - 1$ is, therefore, the probability of survival for a n hit model.

c) Plot of the data of 7a as log (% survival) as a function of coelomic fluid volume. The curve fits the multiple-hit models of 7b and the intercept on the y axis lies between that of a 3- and 4-hit model.

can be calculated for single and multiple hit models on the assumption that the chance of a molecule of GAF interacting with a bacterium is governed by Poissonian probability distribution. Such an approach has been used extensively in analyzing complement hemolytic (Mayer, 1961) and bactericidal action (Wright and Levine, 1981b). A comparison of theoretical-to-actual dose-response curves again indicates that a model requiring 3 or 4 hits/bacterium provides the best fit to the data (Figs. 7b, c). Since both initial binding rate data and dose-response curve data can theoretically be explained by a model requiring several different components of the CF to act cooperatively to kill a bacterium, this possibility cannot be conclusively dismissed.

The data discussed so far were all generated using the turbidometric assay of bacterial killing. In contrast, the plate assay gives a measure of the rate at which bacteria were actually killed (*i.e.*, the combined rate of both GAF binding and killing reactions). Furthermore, since binding is relatively fast, the rate-limiting (and hence, rate-determining) step is the second one, and the plate assay actually measures the rate at which bound GAF causes an irreversible change in the bacterium leading to cell death. The initial rate increases with GAF concentration at low concentrations (Fig. 6b), but levels off at higher concentrations. This limiting velocity, presumably determined solely by the rate of the physiological reaction causing the death of the bacteria, can be calculated by plotting a double-inverse plot of the rate versus concentration (the Lineweaver/Burke plot of enzymologists), as shown in Figure 6c. The limiting velocity obtained by this method is approximately 5×10^5 bacteria/minute, or approximately 80% of the bacteria per minute.

The rate of killing continues to increase at concentrations several times larger than the minimum required to obtain 95% bacterial killing (Fig. 6a), implying that extra molecules of GAF speed up the rate at which a bacterium is killed.

Bacterial killing rate is considerably inhibited at room temperature versus 37°C. One possible explanation for this phenomenon is that the action of GAF requires some metabolic reaction to take place. Alternatively, GAF activity may be influenced by the physical state of the bacterial cell membrane, which is known to vary substantially at different temperatures (Stein *et al.*, 1969; Melchior *et al.*, 1970; Jacobson, 1976).

A third possibility, namely, that GAF action requires the bacteria to be actively dividing in order to be effective can be dismissed since bacteria are routinely killed while still in stationary phase (see, for example, Fig. 3). If the optimal killing temperature for GAF depends on properties of the target organism, such as membrane fluidity, it will be necessary to extend these experiments to strains of bacteria isolated from the immediate natural environment of *Glycera*. Perhaps GAF activity against such organisms would be optimal at temperatures more physiological for both polychaete and bacterium. Such experiments will also be of value in assessing more fully the role of GAF in the natural defense systems of *Glycera*.

It does not appear that GAF is identical to other antibacterial factors found in invertebrate sera, although few of these have been studied in sufficient detail to permit detailed comparison. GAF does not possess any of the properties of the lysozyme-type enzymes which have been described in a number of other bactericidal systems (Powning and Davidson, 1973; Cheng and Rodrick, 1974; Jollès and Jollès, 1975; Cheng *et al.*, 1977; Anderson and Cook, 1979; Cheng, 1980). Unlike lysozyme, GAF does not lyse *Micrococcus luteus* cell walls and is most active against Gram-negative bacteria (Anderson and Chain, 1982). It is also quite distinct from the inducible bacteriolytic proteins which have been recently isolated from the hemolymph of lepidopteran pupae (Hültmark *et al.*, 1980). There are some mechanistic similarities, however, between GAF and vertebrate complement. The antibacterial action of complement has recently been reexamined in detail (Wright and Levine, 1981a, b). Like GAF, complement action involves an initial binding reaction to the cell surface, followed by a killing reaction (Levine *et al.*, 1953; Muller-Eberhard, 1968); it has a low multiplicity, probably requiring two "killing units" per bacterium (Wright and Levine, 1981b); it is highly temperature-sensitive (Mayer and Levine, 1954), and has an absolute requirement for divalent cations (Levine *et al.*, 1953; Muschel and Treffers, 1956b). At the other end of the evolutionary spectrum, similar characteristics are shown by certain types of colicins (*e.g.*, K1 and E1), which are bactericidal proteins of bacterial origin (Weiss and Luria, 1978; Wendt, 1970). It is extremely unlikely that there is any true homology between GAF and either complement or the colicins. Rather, the similarities probably arise from parallel strategies evolved in these diverse organisms to eliminate competing or pathogenic microorganisms.

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ANTIBACTERIAL ACTIVITY OF THE COELOMIC FLUID FROM THE POLYCHAETE, *GLYCERA DIBRANCHIATA*.
II. PARTIAL PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF THE ACTIVE FACTOR

BENJAMIN M. CHAIN AND ROBERT S. ANDERSON

Sloan-Kettering Institute for Cancer Research, Donald S. Walker Laboratory,
145 Boston Post Rd., Rye, New York 10580

ABSTRACT

The coelomic fluid of *Glycera dibranchiata* is bactericidal for the Gram-negative rod, *Serratia marcescens*. An active factor has been partially purified using gel chromatography and affinity chromatography on concanavalin A-Sepharose, and has been shown to be a glycoprotein with a molecular weight of $2.5–4.5 \times 10^5$ containing bound divalent cations and at least one disulfide bridge. The active molecule showed no biochemical similarities to other humoral bactericidal systems, either vertebrate or invertebrate, and would appear to define a new class of antibacterial molecules.

INTRODUCTION

We have previously described a bactericidal factor in the coelomic fluid of the polychaete *Glycera dibranchiata* (Anderson and Chain, 1982), and have studied the kinetics of its action against the Gram-negative rod, *Serratia marcescens* in the previous paper. These studies suggested, *inter alia*, that *Glycera* antibacterial factor (GAF) is a heat-labile protein, which acts by a 2-step process involving an initial reversible binding to the bacterial cell surface, followed by a "killing reaction" which perhaps involves damage to the cell membrane. In this paper we present some studies on the biochemical characterization of GAF. Only a partial purification of GAF has been achieved and many details of its structure remain unknown. However, the information which has been gained is sufficient to allow us to begin to draw comparisons with other humoral bactericidal systems.

METHODS

Coelomic fluid from *Glycera dibranchiata* was obtained as described previously and antibacterial activity was measured using a turbidometric assay (Chain and Anderson, 1982). Briefly, coelomic fluid (CF) and bacteria (6×10^6 organisms from an overnight culture of *S. marcescens*) were incubated together in 0.5 ml artificial sea water (ASW) at room temperature for 30 min. 4 ml of tryptic soy broth (TSB) were then added to each tube, and the bacteria grown up for 4–5 h at 37°C. The optical density of the cultures (compared to controls without CF) could be converted directly to % bacteria killed.

Column chromatography was carried out at 4°C using gels obtained from Pharmacia Fine Chemicals; protein concentration in the eluants was continuously monitored by recording UV absorbance (280 nm). Gel filtration was carried out on 50 or 100 × 1.5 cm columns of Sephacryl S-300, molecular weight range 10^4–1.5

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$\times 10^6$, or Sepharose 4B, molecular weight range 6×10^4–20×10^6. Ion-exchange chromatography was carried out on DEAE-Sepharose-C1 6B. Coelomic fluid was dialyzed, using Spectrapor 2 tubing (Spectrum Medical Industry, Inc.), overnight against a large volume of Tris buffer (pH 8.5, 0.1 *M*), containing 10 m*M* calcium chloride. 3 ml of dialyzed coelomic fluid were run onto the column and unbound material was washed off using the same buffer. Elution was carried out with a linear 0–0.5 M gradient of sodium chloride in the same buffer. Affinity chromatography was carried out on a 8 × 1.5 cm column of Con A–Sepharose 4B. Coelomic fluid (10–20 ml) was run through the column, which was then washed with several volumes of ASW. To elute bound components a volume (equal to the void volume of the column) of a 0.5 *M* solution of 1-O-methyl α-D-glucopyranoside or α-methyl D-mannoside in ASW was run onto the column and left for 30 min to insure that all bound components were displaced by the sugar. The displaced glycoprotein was eluted with additional 0.5 *M* saccharide solution. Column fractions were concentrated for electrophoresis using immersible CX-30 Millipore filters (nominal M.W. retention >30,000).

Gel electrophoresis was carried out at 120 volts for 16 h on Pharmacia precast polyacrylamide gradient gels; gels useful in the molecular weight range 5×10^4–2×10^6 and 10^5–5×10^6 were used. Gels were stained for protein with Coomassie blue (1 h, 0.25%) or for glycoproteins using the periodate-Schiff stain according to the protocol of Smith (1976).

Artificial sea-water was made up using Instant Ocean (Aquarium Systems) to a total salinity of 38‰. Its ionic composition has been given previously (Chain and Anderson, 1982). Other chemicals were obtained from Sigma Chemical Company.

RESULTS

Preservation of GAF activity

Two major problems have hindered the biochemical purification of GAF: the greatly accelerated loss of antibacterial activity occurring upon CF dilution, and the rather rigid ionic requirements of the active moiety. The effects of overnight dilution of CF in ASW (the optimum ionic medium for preserving GAF activity) are shown in Table I. As can be seen, substantial activity was irreversibly lost in 16 hours if CF was diluted 1:50–1:100. In contrast, other studies showed that undiluted CF retained full activity at 4°C for 2–3 days. The phenomenon of loss of activity on dilution was particularly apparent in the gel filtration experiments reported below. Various attempts at preserving the activity by inhibiting disulfide bond formation

TABLE I

Effects of dilution in artificial sea water on coelomic fluid activity

	Antibacterial activity (% killing)	
Volume of coelomic fluid (μl)	Diluted immediately before assay	Diluted 16 hours before assay
5	53 ± 12	3 ± 3
10	98 ± 1	70 ± 5

Antibacterial activity was measured using the turbidometric assay with a 30 min initial incubation period. The specified volume of coelomic fluid was diluted into 0.5 ml ASW for the assay. Results are expressed as % killing ± S.E.M., N = 3.

TABLE II

Antibacterial activity of Glycera *coelomic fluid after dialysis against solutions of differing ionic composition*

Dialysis solution	Activity (μl CF required for 50% decrease in optical density)
Artificial Sea Water	5.0 ± 0.4 (3)
10 m*M* $CaCl_2$ in Tris Buffer (0.01 *M*, pH 8)	6.5 ± 0.7 (6)
10 m*M* $MgCl_2$ in Tris Buffer (0.01 *M*, pH 8)	13.0 ± 2.6 (3)
1 *M* NaCl in Tris Buffer (0.01 *M*, pH 8)	6.0 ± 1 (3)
Tris Buffer (0.01 *M*, pH 8)	no activity
Phosphate Buffer (0.05 *M*, pH 7.5)	no activity
1 *M* NaCl + 1 m*M* EDTA in Tris Buffer (0.01 *M*, pH 8)	no activity
Undialyzed coelomic fluid	4.6 ± 0.5 (11)

1–2 ml of coelomic fluid was dialyzed overnight against 1 liter of the solutions indicated above. Various volumes of retentate were assayed after dilution in ASW for bactericidal activity, using the turbidometric assay, and the volume required for a 50% decrease of optical density was measured from the dose-response curve obtained. Results are given as $\bar{X}$ ± S.E.M., (N).

(—SH oxidation) with dithiothreitol, by introducing a protein carrier (BSA), or by cross-linking with very low concentrations of gluteraldehyde, were unsuccessful. Dithiothreitol totally destroyed GAF activity suggesting the presence of disulfide bonds in the GAF molecule.

Experiments illustrating the ionic requirements for preservation of GAF activity are shown in Table II. Overnight dialysis against solutions without divalent cations (Tris or phosphate buffers, or 1 *M* sodium chloride with 1 m*M* EDTA) irreversibly destroyed GAF activity. Native GAF activity was preserved by dialyzing against ASW. As little as 10 m*M* $CaCl_2$ preserved most of the activity, while the same concentration of $MgCl_2$ was less effective. High concentrations of sodium chloride were also sufficient even in the absence of added calcium, perhaps as a result of enhanced binding of intrinsic divalent cations in conditions of high ionic strength.

Although optimal preservation was obtained in ASW, other solutions (containing only the minimum requirement of 10 m*M* calcium chloride) had to be adopted in some experiments in order to use particular techniques, such as ion exchange chromatography, or polyacrylamide gel electrophoresis.

Gel filtration

Elution profiles of coelomic fluid eluted in ASW from two types of gels are shown in Figures 1 and 2. Antibacterial activity after frationation on Sepharose 4B (an agarose gel with an exclusion limit of 2×10^7 Daltons), or with Sephacryl S-300 (an alkyl dextran gel cross-linked with N,N′-methylene bis acrylamide, exclusion limit 1.5×10^6 Daltons) appeared as a single symmetrical peak associated with the major protein peak. A comparison of the elution volume of the peak of antibacterial activity to known protein standards gave molecular weight values of $4–5 \times 10^5$ Daltons on Sepharose 4B, and $2.5–3 \times 10^5$ on Sephacryl S-300.

Measurement of total protein concentration in each fraction (Lowry *et al.*, 1951) was used to compare the specific activity in the eluate to that in the original coelomic fluid. The fractions showing 90–100% bactericidal activity had a protein concentration of 0.4–0.6% mg/ml. With unfractionated coelomic fluid equivalent activity

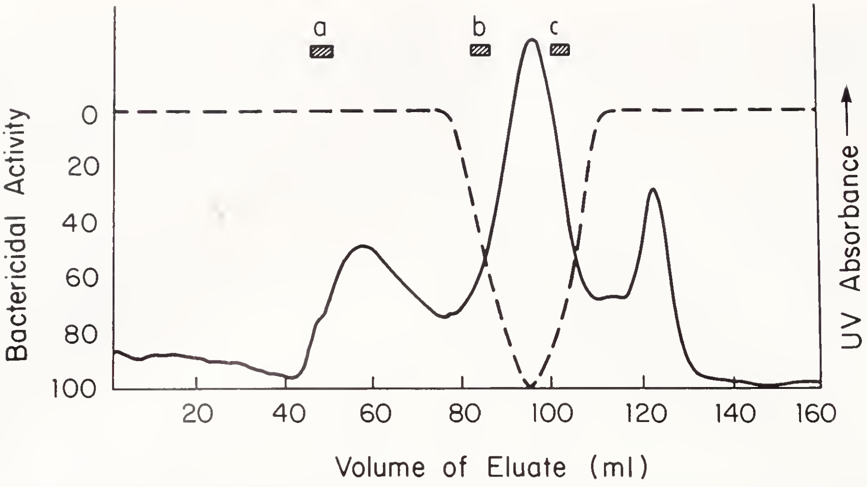

FIGURE 1. Gel filtration of 3 ml coelomic fluid on Sepharose 4B, elution with ASW. Three ml fractions were collected and 0.5 ml of each fraction was tested for bactericidal activity using the turbidometric assay.

a) Void volume; b) Thyroglobulin, M.W. 669,000; c) Aldolase, M.W. 158,000

—— UV absorbance (280 nm) – – – Bactericidal activity

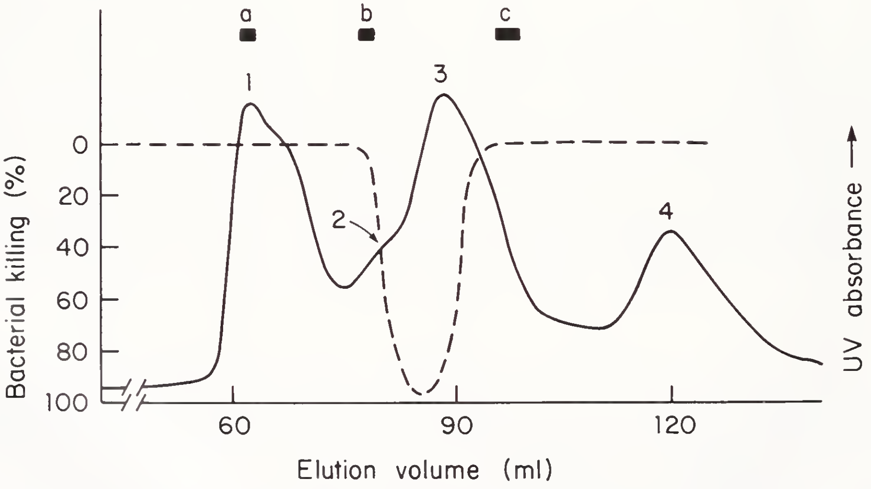

FIGURE 2. Gel filtration of concentrated coelomic fluid on Sephacryl S-300, elution with ASW. Fifteen ml of CF were concentrated to 4 ml by pressure ultrafiltration through Millipore Pellicon membrane ultrafilters (type PTHK). Three ml fractions were collected and 0.5 ml of each fraction was tested for bactericidal activity using the turbidometric assay. Numbered peaks are analyzed by electrophoresis as shown in Figure 3.

a) Void volume; b) Ferritin, M.W. 440,000; c) Aldolase, M.W. 158,000

—— UV absorbance (280 nm) – – – Bactericidal activity was obtained with a protein concentration of 0.1–0.2 mg/ml, indicating a loss in specific activity of GAF during fractionation. One obvious possibility was that GAF was in reality composed of several different components, and that loss of activity was actually a direct result of their separation during purification. However, numerous attempts to reconstitute activity by combining fractions, both from within given peaks and from different peaks, were unsuccessful. It seemed probable, therefore, that a real loss in the biological properties of the active molecule occurred during gel filtration. In part this may be a consequence of protein dilution as was discussed above, and the degree of loss of activity was also correlated to the length of time the GAF remained on the column.

The fractions corresponding to the major protein peaks from the Sephacryl S-300 column were analyzed further by gel electrophoresis on polyacrylamide gradient gels which separate proteins of >150,000 Daltons (Fig. 3). As can be seen, each peak is made up of several components, and overlap is particularly high between peaks 2 (a shoulder on peak 3) and 3. Comparison of biological activity with S-300 protein peaks (Fig. 2) suggests that peak 3 contains the major portion of GAF activity. The main protein components of peak 3 correspond to the major protein band (A) of CF (Fig. 3) and the band just above it on the gel.

We attempted to localize GAF activity directly on gels after CF electrophoresis. Antibacterial activity was visualized by overlaying the gel with trypticase soy agar containing a suspension of *Serratia marcescens* followed by 18 h incubation at 37°C. Bactericidal activity appeared as areas of sparse growth in the otherwise uniform bacterial lawn (Fig. 4). Rather surprisingly, two zones of activity could be seen, one corresponding approximately to the major protein band (A) and the band imme-

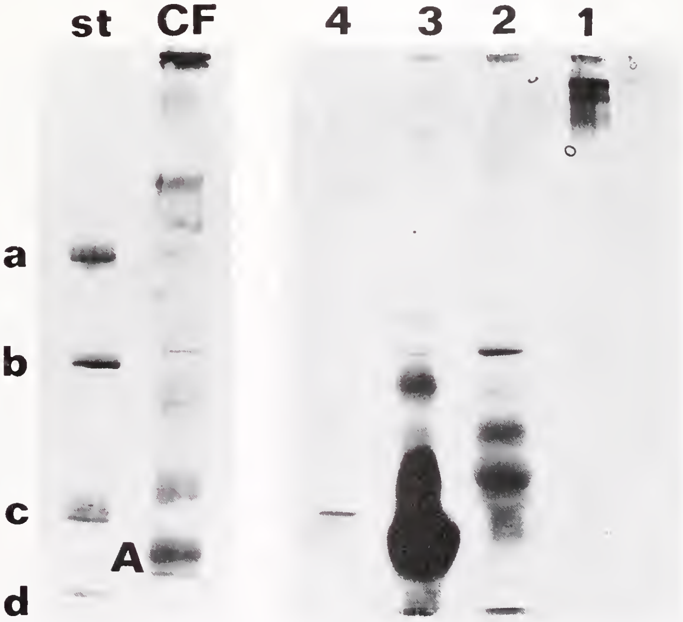

FIGURE 3. Polyacrylamide gradient gel electrophoresis of coelomic fluid (CF) and major protein peaks (1–4) of Sephacryl S-300 eluate. Numbers correspond to fractions shown in Figure 2. St = Standards a) Thyroglobulin, M.W. 669,000; b) Ferritin, M.W. 440,000; c) Catalase, M.W. 232,000; d) Lactate dehydrogenase, M.W. 140,000. A = Major protein band in CF.

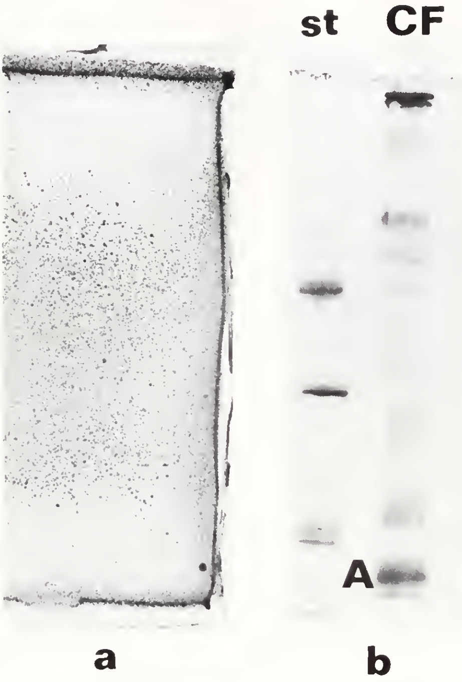

FIGURE 4. Antibacterial activity in coelomic fluid (CF) visualized after electrophoretic fractionation on a polyacrylamide gradient gel. a) Area of a gel overlayed with bacteria (6×10^5 *S. marcescens*/ml in TS agar). b) Portion of the same gel stained for protein with Coomassie blue. Protein standards (st) are the same as used in Figure 3; A = Major protein band of CF.

diately above it, and the other to a very high molecular weight component. It seemed likely that this second band of inhibition resulted from aggregation of GAF which occurs under experimental conditions and probably corresponds to S-300 protein peak 1.

Other purification methods

Ion exchange chromatography on DEAE-Sepharose anionic columns (at pH 8.5) was carried out using linear ionic strength elution gradients. Although excellent separation of several components was obtained, no activity could be recovered. Once again this could not be attributed to separation of two or more components required for activity since elution of all components simultaneously (using a one-step elution change from low ionic strength to ASW) still resulted in total loss of activity. Simple passage through the column at ionic strengths too high to allow binding had no effect on activity. It would appear that the interactions occurring during binding of coelomic fluid components to the column or during dilution, were sufficient to permanently destroy the bactericidal activity.

A more successful approach was affinity chromatography on a Con A Sepharose 4B column (Fig. 5). The majority of the coelomic fluid components failed to bind to the column and could be eluted with ASW. However, all antibacterial activity was adsorbed onto the column. A fraction eluted from the column with a high concentration of α-methyl D-glucopyrannoside (or mannoside) showed a bactericidal effect. However, this activity represented only a minute fraction of the total

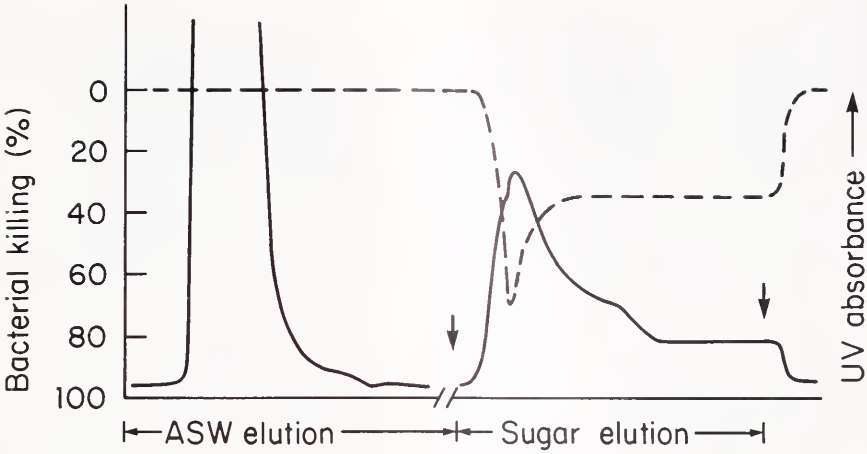

FIGURE 5. Affinity chromatography of CF on Con A-Sepharose 4B. Ten ml of coelomic fluid were run through a short (8 × 1.5 cm) column of Con A-Sepharose using artificial sea-water as the eluent. At arrow eluent was changed to a 0.5 *M* solution of 1-0-methyl-α-D-glucopyranoside in ASW. Three ml were run onto the column and flow was stopped for 30 min to allow time for the competing ligand to displace substances bound by the Con A. Glycoproteins were then eluted with more sugar solution. At second arrow eluent was changed back to ASW. Three ml fractions were collected throughout the experiment and 0.5 ml of each fraction tested for bactericidal activity using the turbidometric assay. Note that the high sugar concentration alone inhibited bacterial growth to a certain extent.

—— UV absorbance (280 nm)

– – – Bactericidal activity

activity put onto the column, either because the active factor remained bound to the column, or because it had been irreversibly denatured by binding to the column.

Figure 6a shows an analysis of the Con A fractionation using polyacrylamide gradient gel electrophoresis. Although Con A selectively removes certain high molecular weight components of the coelomic fluid, most of the major proteins in the region of band A are not retained, suggesting that GAF is quantitatively a very minor constituent of CF proteins of this molecular weight. However, electrophoresis of a concentrate of the material initially bound to Con A, and subsequently eluted with methyl glucose, showed that this fraction indeed included a small component of the major band (A). Since Con A selectively binds glycoproteins, samples of whole CF and the Con A binding fraction were also stained with the PAS glycoprotein stain (Fig. 6b). The PAS-positive bands in whole CF correspond rather closely to those bound to Con A. The only apparent anomaly is that the Con A eluate component of band A is apparently PAS-negative, though there is a PAS-positive component of whole CF with the same molecular weight. The concentration of this component in the Con A eluate may be too low to stain with the PAS stain, which is less sensitive than Coomassie blue.

DISCUSSION

Glycera antibacterial factor appears to be a glycoprotein of molecular weight ~2.5–4.5 × 10^5. There are also strong indications that the molecule contains bound divalent cations which are essential to its structural integrity and at least one disulfide bridge. Because of the loss in bactericidal activity, which occurred during all chromatographic separations, and the presence of multiple components in column frac-

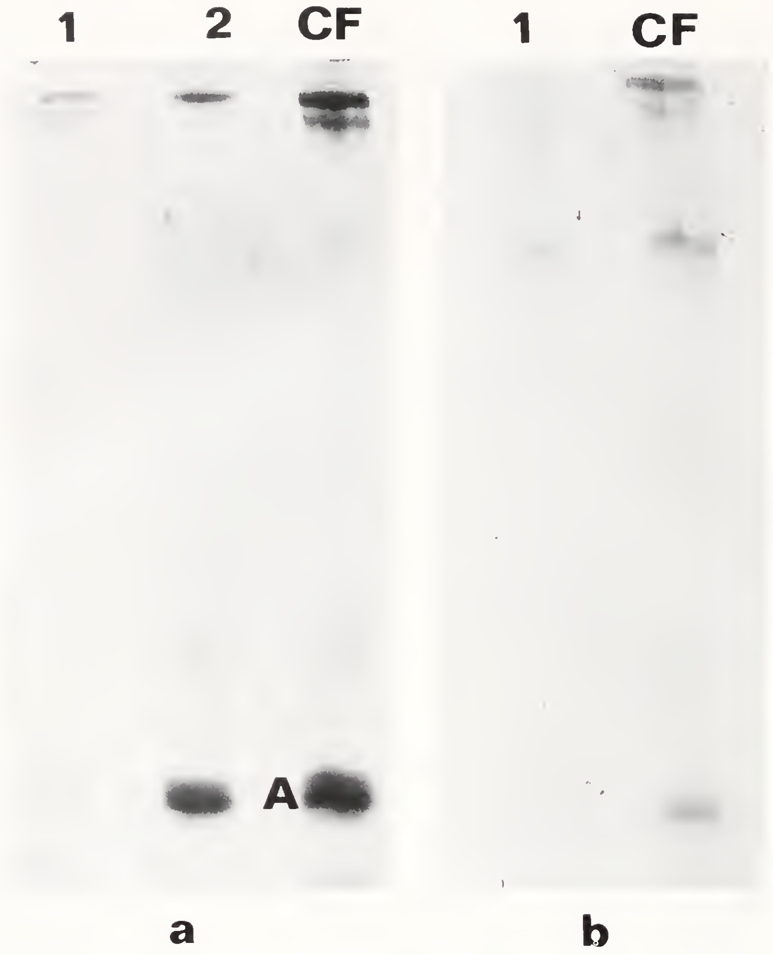

FIGURE 6a. Analysis of affinity chromatography experiment (see Fig. 5) using polyacrylamide gradient gel electrophoresis. Gel stained for protein with Coomassie blue. CF = untreated coelomic fluid; 1 = CF components bound by Con A and eluted with 0.5 *M* 1-0-methyl-α-D-glucopyranoside; 2 = CF after passage through Con A column; A = major protein band of CF.

b. Gel stained for glycoproteins using periodate-Schiff reagent. 1 = PAS staining of CF components bound by Con A column and eluted as in Figure 6a.

tions even after purification, as shown by gel electrophoresis, it is as yet impossible to rule out the possibility that several different molecules have GAF activity, or that GAF aggregates may exist. Therefore, the results obtained may only refer to one of the major antibacterial components of the coelomic fluid. The molecular weight estimates of GAF clearly differentiate it from any other antibacterial system found in either vertebrates or invertebrates. For example, it is clearly different from bactericidal proteins recently isolated from insects (Hültmark *et al.*, 1980) and oligochaetes (Roch *et al.*, 1980). Nor is there any evidence of any relationship to components of the vertebrate complement system (Müller-Eberhard, 1968; Müller-Eberhard and Schreiber, 1980), although the bactericidal mechanism of action of the two systems may show some similarities. In view of the probable cytotoxic, as well as bactericidal activity of GAF (Chain and Anderson, unpublished observation), it may also be of interest to compare it to the lymphotoxin system found in vertebrates. The most active component(s) of lymphotoxin from both human (Harris *et al.*, 1981) and various other animal lymphoid cells (Ross *et al.*, 1979) has a molecular weight in the same range as GAF, and will also readily aggregate or break down into component parts with little or no biological activity.

It would be of great interest to dissect further the component parts of the GAF molecule, particularly in relation to separate functions such as cell binding and recognition, and cell killing. However, these studies will have to await improvements in the biochemical and functional analysis of GAF.

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MARGINAL BANDS OF LOBSTER BLOOD CELLS: DISAPPEARANCE ASSOCIATED WITH CHANGES IN CELL MORPHOLOGY

WILLIAM D. COHEN, IRIS NEMHAUSER, AND MARION F. COHEN

Department of Biological Sciences, Hunter College, 695 Park Ave., New York, NY 10021, and The Marine Biological Laboratory, Woods Hole, MA 02543.

ABSTRACT

Blood cells of the lobster *Homarus americanus* were examined for the presence of marginal bands of microtubules (MBs*). Nearly all of the cells were highly flattened and elliptical immediately after removal from the animals, and they contained cytoplasmic granules. Rapid cell lysis with Triton X-100 under microtubule-stabilizing conditions produced granule-free "clots" in which almost every nucleus had an associated MB. Thin sections of intact, oriented cells revealed MBs of 50–60 microtubules in classical location in the plane of cell flattening, close to (but not touching) the plasma membrane. Cells rapidly lost native morphology as they spread on glass substrata. The fate of the MB during spreading was followed in lysed cell "cytoskeletons" under phase contrast and by means of indirect anti-tubulin immunofluorescence. MBs initially appeared twisted, then splayed apart, and finally disappeared completely after cell spreading for 20 min. These observations and previous literature support identification of the MB-containing cells as the clotting cells of *Homarus*, in which the *in vitro* behavior may reflect a self-destructive *in vivo* mechanism. Their similarity to clotting cells of other species is discussed.

INTRODUCTION

The comparative hematology of invertebrates has received relatively little attention since the publication of Andrew's text (1965), but the recent comprehensive survey by Ratcliffe and Rowley (1981) has helped to renew interest in the fascinating array of invertebrate blood cell types. It is evident, however, that the study of invertebrate blood cells is somewhat inhibited by the confusing terminology with which they are described. While they are often referred to in general as "hemocytes" which exist in "hemolymph", their specific classification has been made by various authors on different bases such as staining properties ("basophils", etc.), morphological features ("granulocytes", etc.), motile behavior ("amebocytes"), and anatomical source ("coelomocytes"). Additional complexity arises from the tendency of certain cell types to undergo rapid changes of morphology after removal from the animal. Thus a population of cells which are initially fairly uniform in shape may exhibit a diversity of shapes in cytological preparations.

These problems are quite apparent in studies of the blood cells of the lobster, *Homarus americanus*. Three papers describing the hemocyte types in this species (Toney, 1958; Hearing and Vernick, 1967; Cornick and Stewart, 1978) differ with respect to the basis of their specific classification, and thus their data are difficult to correlate. Moreover, *Homarus* hemocytes undergo dramatic shape changes sub-

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* Abbreviations: MB = marginal band, EGTA = ethyleneglycol-bis (β-aminoethyl ether) N, N′-tetra acetic acid, TAME = p-tosyl arginine methyl ester HCl, PIPES = piperazine-N-N′-bis (2-ethane sulfonic acid)

sequent to removal from the animal. Most of the cells are initially flattened and elliptical, but rapidly lose their ellipticity and assume various shapes while spreading thinly on substrata (Toney, 1958).

With respect to their initial flattened, elliptical morphology, lobster hemocytes are similar to all non-mammalian vertebrate erythrocytes (Andrew, 1965) and to *Limulus* amebocytes (Armstrong, 1979), all of which contain marginal bands of microtubules (MBs) as part of their cytoskeletal system (Fawcett and Witebsky, 1964; Goniakowska-Witalinska and Witalinski, 1976; Nemhauser *et al.*, 1980). The purpose of this study was to determine whether lobster blood cells also contain MBs, and, if so, whether alterations of MB structure accompany *in vitro* changes in cell morphology.

Materials and Methods

Lobsters (*Homarus americanus*) were maintained in running sea water or in cooled (15°C), aerated tanks of "Instant Ocean" artificial sea water (Aquarium Systems, Inc.). Blood (hemolymph) was obtained from the animal by rapidly snipping a walking leg and drawing the exuded fluid into a Pasteur pipette. Fresh samples were used for each experiment. When required, marine crustacean Ringer's solution, pH 7.0 (Cavanaugh, 1975), was used as a blood diluent. Cell morphology was examined via phase contrast microscopy of both living cells and cells fixed in Ringer's containing glutaraldehyde (1% or 0.1% equally effective). Fixation was particularly valuable since it prevented changes of morphology during handling and observation of the material.

For thin sectioning, fresh blood was immediately diluted approximately 1:10 into fixative consisting of 9 volumes marine crustacean Ringer's solution and 1 volume 10% glutaraldehyde. Samples were then placed onto polylysine-coated plastic coverslips (Bel-Art Inc.). The coverslips had been pre-treated for 15 min with a 1% solution of polylysine of MW > 400,000, followed by water washes and drying. The samples were incubated on these coverslips in a moist chamber for 1 h at room temp., during which time many of the fixed cells adhered to the polylysine substrate with their plane of flattening approximately parallel to that of the coverslip. Following washes in Ringer's and in 0.1 *M* phosphate buffer, pH 6.9, the adhering material was post-fixed in 1% OsO_4 in the same buffer for 1 h. The coverslips, with adhering material, were then washed in phosphate buffer, dehydrated in an ethanol series, infiltrated with Epon-ethanol (to avoid possible dissolution of the plastic coverslip in propylene oxide), and flat-embedded in Epon. The coverslips were subsequently peeled away from the Epon, and some embedments were glued flat onto the tips of Epon capsule blanks for sectioning approximately parallel to the plane of cell flattening. Other embedments were recoated with Epon to sandwich the cells, and oriented such that sections were cut approximately perpendicular to the plane of cell flattening. Orientation of the cells was surveyed under phase contrast by means of 2 μm thick sections. Thin sections were cut with a diamond knife on the MT-2 ultramicrotome (DuPont-Sorvall Instruments), stained with saturated uranyl acetate in 50% ethanol followed by Reynold's lead citrate, and examined in the Hitachi HS-8 transmission electron microscope operating at 50 kV.

For rapid and convenient testing for the presence of MBs, cells were lysed with Triton X-100 under microtubule-stabilizing conditions. The lytic medium consisted of 1 m*M* $MgCl_2$, 5 m*M* EGTA*, 10 m*M* TAME, 100 m*M* PIPES, 0.4% Triton X-100, pH 6.8 with KOH. This medium (="LyM") has been employed previously to reveal MBs in both vertebrate and invertebrate blood cells (Cohen, 1978; Nemhauser *et al.*, 1980).

Indirect immunofluorescence after anti-tubulin binding was also used to visualize MBs during cell spreading. Fresh blood was immediately diluted approximately 1:10 into Ringer's and "zero-time" cytoskeletons prepared by 1:10 lysis in LyM. These cytoskeletons were found to adhere to glass coverslips which had been pre-cleaned with acid, after about 10 min incubation. Living cells were allowed to attach and spread on coverslips at room temperature (approx. 22°C) for 5, 10, and 20 min, during which time they were kept covered with Ringer's. To prevent the possible accumulation of secreted material during spreading, the coverslips were placed periodically in beakers with large volumes of Ringer's solution. At the appropriate times, the coverslips were drained briefly and the cells lysed by flooding with LyM. They were then rinsed with LyM lacking Triton and TAME, and fixed for 20 min in the same medium containing 3.7% (w/w) formaldehyde. After washing in the same medium without formaldehyde, followed by 3 washes in phosphate-buffered saline (PBS), the material was incubated with anti-tubulin for 30 min at 37°C. It was then rinsed again with PBS and similarly incubated with fluorescein-labelled goat anti-rabbit IGG. After washing in PBS, the coverslips were mounted in PBS for observation. The anti-tubulin was a gift of Dr. R. D. Goldman, then of Carnegie Mellon University. Anti-tubulin specificity had been verified by double immunodiffusion against 3×-cycled mammalian brain tubulin, by the ^{125}I-protein A binding procedure against α and β tubulin, and by using preimmune serum as a control (R. D. Goldman, personal communication). Zeiss phase contrast microscopes equipped with epifluorescence illumination were used throughout. Images were recorded on Plus-X or Tri-X film.

RESULTS

When fresh blood samples were examined immediately in phase contrast or subsequent to dilution into Ringer's containing glutaraldehyde, the predominant cell type observed was highly flattened and elliptical (Fig. 1a). Ellipticity varied from slight (*i.e.* nearly circular, as in Fig. 1b) to extensive (elongate), with the long axis of the ellipse typically in the range of 15–20 μm. Cells of other morphology were present in lesser numbers, as illustrated in Figure 2. Counts were made of different cell types in random fields for a sample from one animal. Of 100 cells counted, there were 71 granular elliptical cells (category A) and 9 with one end oval and the other tapered and refractile (category B). Careful observation of cells in flow under the coverslip showed that the latter cell type was identical to the former except that one end was twisted relative to the other, and thus this morphological type represented about 80% of the population. Some of the remaining cells had one or two sharply pointed ends (category C), and others were generally elliptical in outline but had bulges or constrictions (category D). Many of the celi shapes in these minority categories (Fig. 2) appeared to be variations on a basic flattened elliptical form, so that the 80% figure is probably minimal. Most of these cells contained conspicuous granules which ranged from about 0.3 to 1 μm in diameter, as illustrated in Figure 1c.

Rapid dilution of blood samples with lysing medium containing Triton X-100 (LyM) directly upon removal from the animal produced masses or "clots" of lysed cells. Such clots did not contain any of the cytoplasmic granules visible in the intact cells; nuclei were the most conspicuous components (Fig. 3a). At higher magnification nearly all of the nuclei were found to be circumscribed by phase-dense MBs (Fig. 3b). The bands are only about 0.2–0.4 μm thick; careful focussing was, therefore, required to reveal their presence about nuclei at different levels within the "clot". If the material was squashed under the coverslip, many of the MBs became

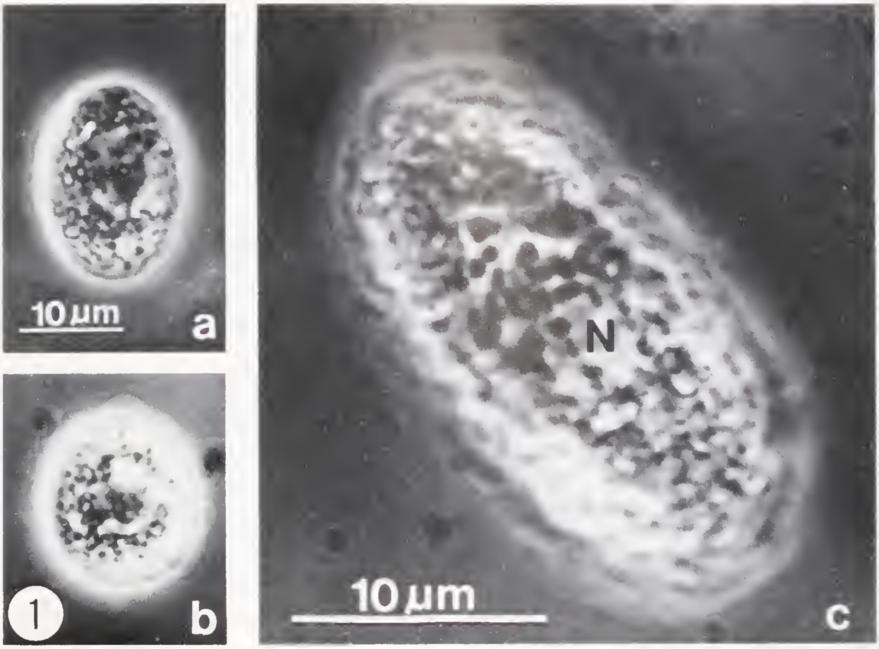


FIGURE 1. *Homarus* blood cells fixed immediately after removal from the animal (in crustacean Ringer's solution containing 1% glutaraldehyde). Nearly all cells were highly flattened; most were elliptical as in (a), but some were more circular (b). The great majority of cells contained conspicuous granules, about 0.3 to 1 μm in diameter. These were most readily visible under oil immersion by focussing in the thinner cytoplasmic layer above or below the nucleus (N), as illustrated in (c). Phase contrast; mag. bar for (b) as in (a).

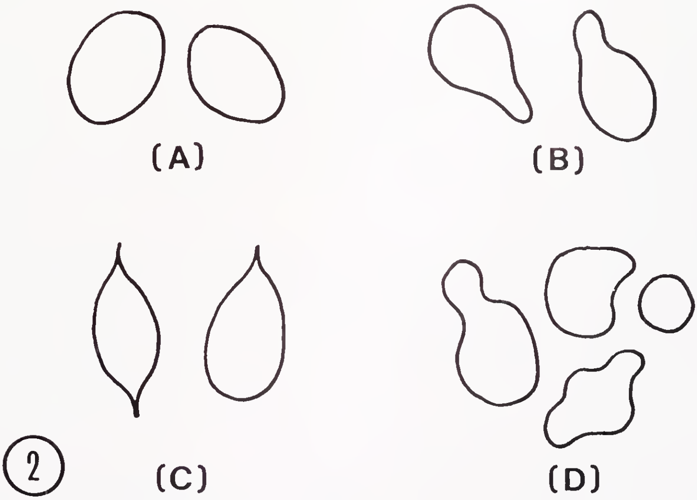


FIGURE 2. Morphology of blood cells fixed immediately after removal from the animal (fixation: 1:10 dilution into Crustacean Ringer's solution containing 1% glutaraldehyde). One hundred cells in non-selected fields were surveyed in phase contrast; the number (= percentage) of cells in each category is given in parenthesis. Morphology category A: cells flattened and elliptical (71); category B: one end flattened and elliptical, other end rounded, tapered, and refractile (9); category C: flattened, elliptical, with a sharp point at one or both ends (6); category D: various other shapes (14).

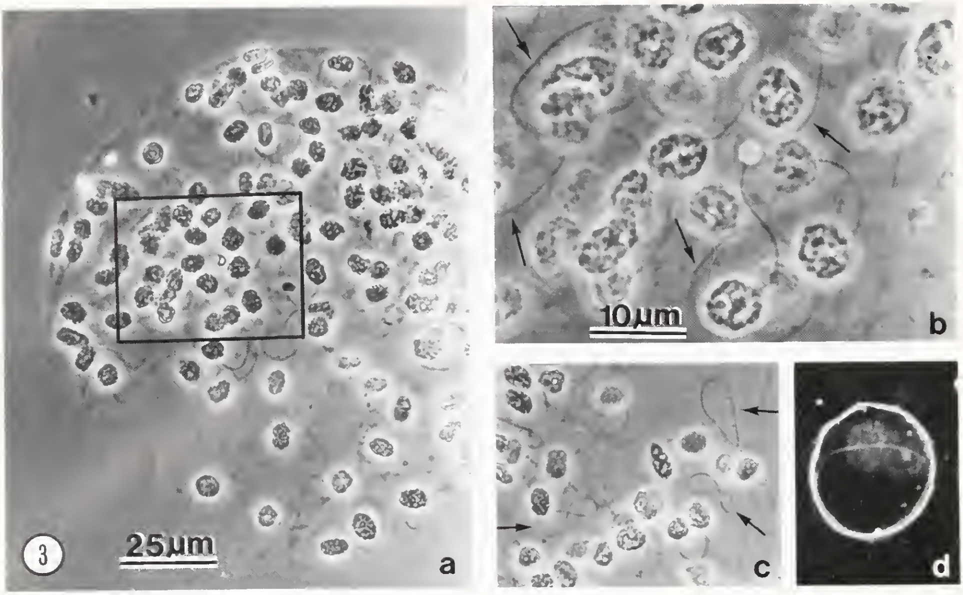


FIGURE 3. Marginal bands of lobster blood cells visualized by direct, rapid dilution of blood sample with lytic medium containing Triton X-100 (LyM). (a) "Clot" of lysed cells produced by 1:10 blood dilution into LyM, in which nuclei are prominent and granules are not present. (b) Higher magnification view of area delimited by the rectangle in (a), showing MBs adjacent to individual nuclei (arrows). (c) Squashed "clot" showing some MBs free of nuclei (arrows). (d) MB of lysed cell as visualized by indirect anti-tubulin immunofluorescence. MB microtubules are brightly fluorescent. In most cells fluorescence was confined to the MB, with a separated microtubule or thin bundle occasionally present, as illustrated. (a–c) Phase contrast; mag. bar for (c) as in (a). (d) Fluorescence microscopy, bar for (d) as in (b).

displaced from nuclei, appearing as free closed loops or figure-8 forms (Fig. 3c). When fresh blood samples were first rapidly diluted 1:10 into crustacean Ringer's solution and then immediately diluted 1:10 again into LyM, large clots did not form, and there were many more individual lysed cells present. These also contained a nucleus circumscribed by the MB, with no visible granules. In preparations treated with anti-tubulin and examined by the indirect immunofluorescence technique, MBs were brightly fluorescent, with few or no microtubules visible except those of the MB bundle (Fig. 3d).

The generality of MB occurrence was examined with respect to lobster size and sex. As shown in Table I, MBs were present in hemocytes of both males and females 12 to 22 cm in length. The impression that nearly all cells contained MBs was verified by counts in non-selected fields which showed that approximately 95% of the nuclei were circumscribed by MBs.

In order to verify the expected location of the MBs in intact cells, and to demonstrate their microtubular substructure directly, cells were thin-sectioned either approximately parallel to or perpendicular to their plane of flattening. In the former case, elliptical or circular cell profiles were generally observed (Fig. 4a, b), most of which displayed prominent marginal bundles of microtubules in longitudinal or somewhat oblique section (Fig. 4c, d). When sections were perpendicular to the plane of flattening, cell profiles were relatively narrow with tapered ends (Fig. 5a), and the cross-sectioned bundle of MB microtubules was present in classical location at opposite ends near the cell surface (Fig. 5b, c). The MB contained about 60–70

TABLE I

Percentage of lobster blood cells containing marginal bands (MBs)[a]

Animal No. and Sex	Length[b] (cm)	Lysed cell counts (20 cells per animal)	
		Nuclei with MBs	Nuclei without MBs
1. ♂	12	20	0
2. ♀	16	20	0
3. ♂	17	19	1
4. ♂	18	18	2
5. ♀	20	19	1
6. ♂	22	18	2
	Totals . .	114	6
	Percentage (of 120) . .	95%	5%

[a] Procedure: sample of hemolymph from snipped leg was diluted approximately 1:20 into marine Crustacean Ringer's solution. The diluted sample was lysed by dilution approx. 1:10 into LyM (immediately!).

[b] Measured from tip of rostrum to tip of uropod.

microtubules, closely but somewhat irregularly packed. It was evident that MB microtubules were not in direct contact with the plasma membrane bilayer at their closest approach, but were separated from it by a layer of material approximately 50 nm thick (Fig. 5b).

Having demonstrated that MBs were present, the question was raised as to the fate of these MBs during morphological transformation of the cells *in vitro*. As observed in phase contrast, most cells were still relatively flat and elliptical one minute after removal from the animal, and in contact with glass (Fig. 6a). Within 5 min the cells became "spiky" and began to spread (Fig. 6b), and after 10 min most cells had spread so extensively that internal organelles began to become visible (Fig. 6c). Granules could be seen disintegrating in such spread cells. At this point, lysis by perfusion with LyM revealed no MBs (Fig. 7a, b).

In order to visualize the MB disorganization sequence, time-course samples were examined by means of anti-tubulin binding and indirect immunofluorescence. Major stages in the sequence are illustrated in Figure 8. At t = 0 (no spreading) the lysed cells contained essentially all of their microtubules within intact circular or elliptical MBs as shown in Figure 3d. After spreading on glass for 5 min, most of the MBs were still recognizable as such. Some were still elliptical (Fig. 8a), but most of them showed signs of disorganization, with figure-8 forms more common than usual (Fig. 8b). After 10 min, recognizable MBs were no longer present, and bundles of microtubules were splayed in various directions (Fig. 8c). By 20 min the microtubule network had disappeared completely, leaving only scattered points of fluorescence visible in some cells (Fig. 8d).

DISCUSSION

Presence of MBs in invertebrate blood cells

With respect to cytoskeletal structure and the presence of MBs, apparently diverse cell types in phylogenetically distant species share a major common feature. There are well-documented reports of MBs in blood cells of Echinoderms (sea cu-

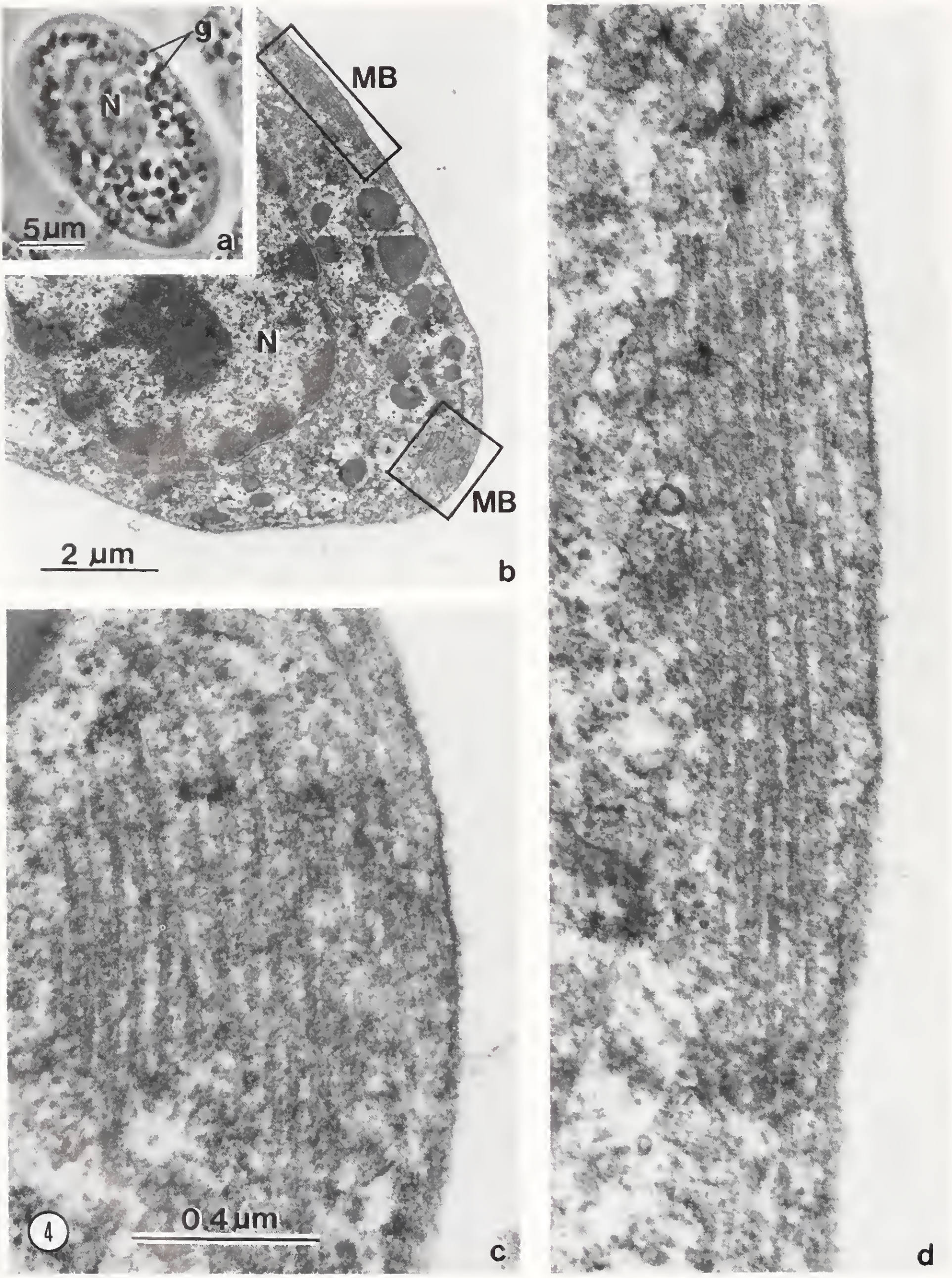


FIGURE 4. *Homarus* blood cells sectioned approximately parallel to the plane of flattening. (a) Thick section (2 μm) in phase contrast, showing general elliptical cell profile with nucleus (N) and granules (g) visible. (b) Transmission electron microscope survey view of part of a thin section comparable to the thick section in (a), with nucleus (N) and cytoplasmic granules visible. The MB appears in two locations at the periphery, as marked by the rectangles. (c, d) Lower and upper rectangles, respectively, of (b), at higher magnification. The MB microtubules are present in longitudinal view, running approximately parallel to the cell surface. Mag. bar for (d) as in (c).

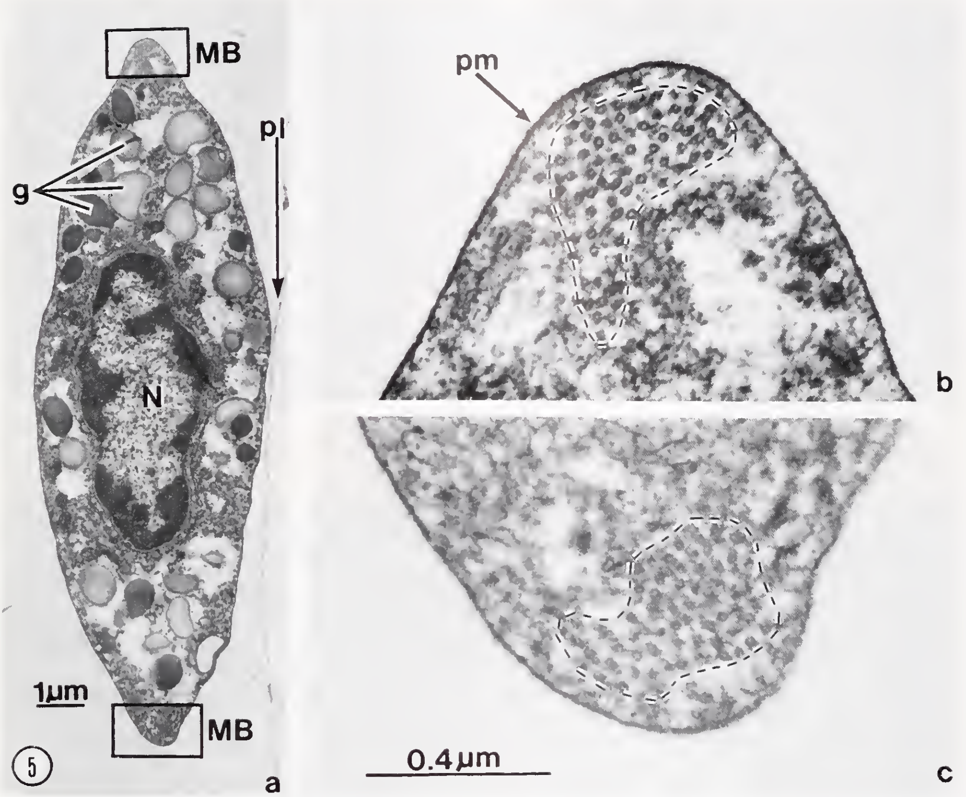
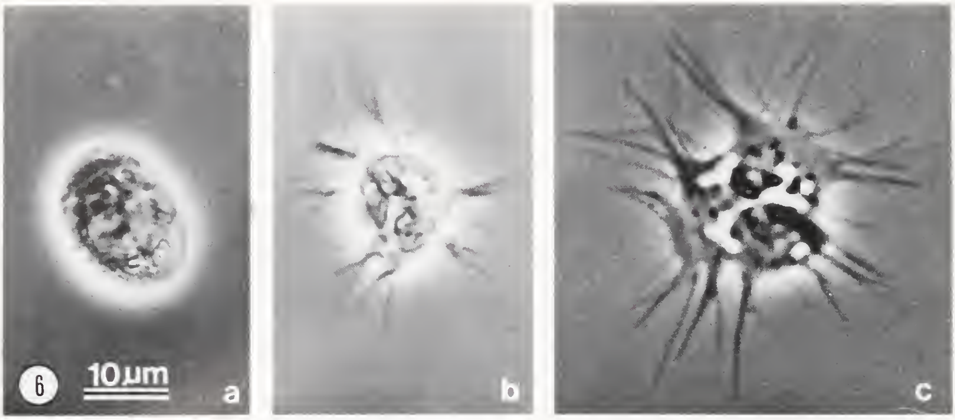


FIGURE 5. *Homarus* blood cell sectioned approximately perpendicular to the plane of flattening (transmission electron microscopy). (a) Complete section of cell, with prominent nucleus (N) and granules (g). The cell lies against the polylysine-coated substratum visible as a vertical dense line (arrow labelled pl). The MB is present in near cross-section in classical location at opposite tapered ends of the cell. (b, c) Higher magnification views of regions delimited by upper and lower rectangles, respectively, of (a). The bundle of MB microtubules is outlined by the dotted line, with 60–70 microtubules present. In (b), where the microtubules are in near cross-section, it is clear that the MB bundle does not directly contact the plasma membrane bilayer (pm), but is minimally separated from it by about 50 nm. Mag. bar for (c) as in (b).

cumber erythrocytes, Fontaine and Lambert, 1972), Sipunculans (*Phascolopsis gouldii* erythrocytes, Nemhauser *et al.*, 1980), Arthropods (cockroach hemocytes, Baerwold and Boush, 1970; *Limulus* amebocytes, Nemhauser *et al.*, 1980; lobster hemocytes, this paper), and Mollusks (erythrocytes of *Anadara* and *Noetia* "blood clam" species, Cohen and Nemhauser, 1980). These invertebrate phyla may thus be added to the vertebrates as represented by all non-mammalian vertebrate erythrocytes, "primitive" mammalian yolk-sac erythrocytes, and mammalian platelets (Fawcett and Witebsky, 1964; Behnke, 1965; van Deurs and Behnke, 1965), illustrating the fact that MBs are a nearly universal (though not necessarily constant) blood cell feature. Although it is possible that MBs may serve different functions in different cell types, it is likely that they share common functions in cellular morphogenesis (Barrett and Dawson, 1974) and in the adaptation of single cells to existence under flow conditions (Cohen *et al.*, 1982).



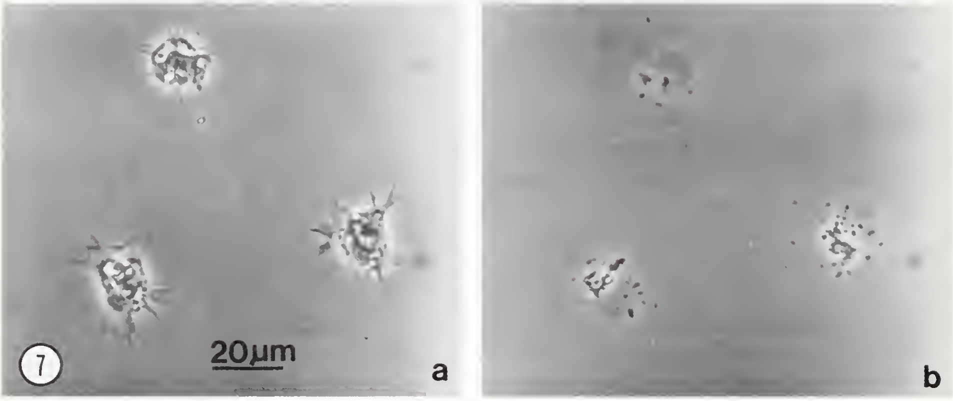


FIGURE 6. Typical spreading sequence for living cells in contact with glass substratum (phase contrast). (a) One min after removal from animal, in contact with glass; cell still elliptical and flat. (b) Same cell after 5 min, becoming amorphous and "spiky". (c) Same cell after 10 min, showing extensive spreading such that internal organelles start to become visible. The three photographs are at the same magnification, with mag. bars as in (a).

FIGURE 7. Three cells allowed to spread for 12 min, before (a) and after (b) lysis by perfusion with LyM. MBs are not present in such spread cells. Phase contrast, mag. bar for (b) as in (a).

Lobster blood cell types

According to Toney (1958), fresh lobster blood (*H. americanus*) observed in phase contrast contains four cell types: relatively small "lymphoid cells" (averaging 11 × 13 µm) and "monocytes" (7 × 10.8 µm), and larger "explosive refractile granulocytes" (16.8 × 25.2 µm) containing either small (.25 µm) or large (1 µm) granules. Nearly all of the cells were round or oval immediately after removal from the animal. The "explosive refractile granulocytes" were identified as active participants in blood clotting, with those containing large granules constituting 90–95% of the total blood cell population. These cells showed extensive morphological changes with time, spreading very thinly, with disintegration of the large granules at their surface producing an "explosive" or "bubbling" effect. Some cells ultimately became almost unrecognizable, with only the nucleus intact. "Explosive refractile granulocytes"

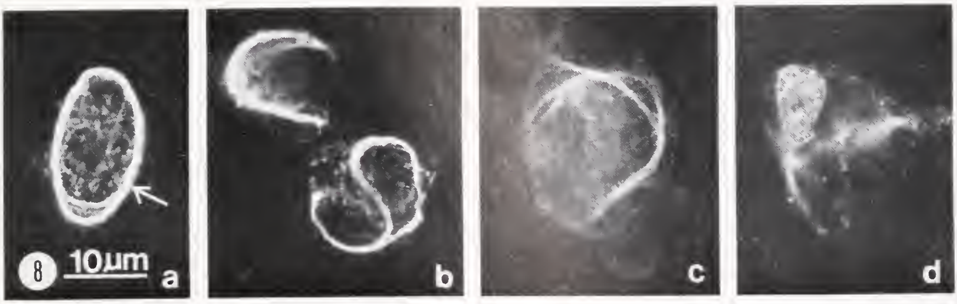


FIGURE 8. Fate of the MB during spreading as visualized by indirect immunofluorescence after anti-tubulin binding. (a, b) Five min spreading; MBs still recognizable as such. A few cells contain elliptical MBs (a, arrow), but in the majority the MBs show signs of disorganization, such as extensive twisting (b). (c) Ten min spreading; MBs are no longer recognizable. Microtubule bundles are evident, and there is a general background fluorescence. (d) Twenty min spreading; there are points of fluorescence visible, but microtubules are not present. The nucleus is also sometimes fluorescent, as in this example; it is not clear whether this is an artifact or may have some significance. Fluorescence microscopy, mag. bars as in (a).

were also described as being the major cell type in the blood of the crayfish (*Cambarus bartoni*) and the blue crab (*Callinectes sapidus*).

Hearing and Vernick (1967) examined the blood cells of *H. americanus* by light and electron microscopy, using samples fixed immediately after removal from the animal. Neither MBs nor microtubules were observed, and apparently they were not preserved by the fixation methods employed. Three cell types were identified and counted with respect to percentage of the population: "Eosinophils" (20% of population), "ovoid basophils" (20%), and "spindular basophils" (60%). All of the cells contained granules, with the largest ones observed in the "spindular basophils". Identification of cell type was based primarily upon the examination of cells in sections, and it appears that the plane of sectioning may not have been taken into account in interpreting the images. Thus it is likely that the "spindular basophils" were flattened, oval cells cut at various angles (as in Fig. 5), and that the "ovoid basophils" were the same cells in face view (Fig. 4). This cell type would then constitute about 80% of the population and contain the larger granules, justifying their identification as the "explosive granulocytes" of Toney (1958) and as the major cell type observed in the present work.

Using stained and unstained films of fixed cells, Cornick and Stewart (1978) described the hemolymph of *H. americanus* as containing four cell types: prohyalocytes (about 2%), hyalocytes (64%), eosinophilic granulocytes (12%), and chromophobic granulocytes (22%). Cells in the last three categories, comprising most of the population, were observed to contain granules of various sizes and staining properties; presumably, most of these cells also correspond to the "explosive granulocytes" of Toney (1958).

It is evident that the phenomenon occurring in the "explosive granulocytes" of the lobster is exocytosis, and that these cells are quite comparable morphologically to *Limulus* amebocytes (Levin and Bang, 1964a, b; Armstrong, 1979, 1980; Ornberg and Reese, 1981). In *Limulus*, all of the clotting factors including clottable protein are believed to be present in the amebocyte cytoplasmic granules (Murer *et al.*, 1975). In *Homarus*, however, fibrinogen has been reported to be a normal component of hemolymph plasma (Stewart *et al.*, 1966), and therefore the granules of *Homarus* blood cells are probably not identical in content to those of *Limulus*.

Homarus hemocyte extracts do initiate clotting (Stewart *et al.*, 1966), and presumably clotting factors are contained in the granules and released during exocytosis. Lysis of *Homarus* granules by Triton in the present work could thus account for the immediate formation of "clots" and the absence of granules from the lysed cells (Fig. 3). As the terminology is confusing with respect to such cells in the Crustacea and for invertebrates in general, we suggest that cells of this type be referred to collectively as "clotting cells". Preliminary observations indicate that MBs are present in similar cells in several species of marine crabs (Cohen *et al.*, 1977), and thus they may be a common feature of Crustacean clotting cells.

MB function

The lobster blood cell MB is a bundle of 60–70 microtubules which is present in classical location near the cell surface in the plane of cell flattening (Figs. 4, 5). As viewed in cross section, the MB microtubules do not make direct surface contact with the plasma membrane bilayer, but are separated from it by a layer of material associated with the cell surface (Fig. 5). Granules observed in thin sections also did not appear to be in direct contact with the bilayer. Gaps between granules and the plasma membrane bilayer have been well-documented in *Limulus* amebocytes prior to activation of exocytosis (Ornberg and Reese, 1981), and the MB microtubules of this species are similarly separated from the plasma membrane bilayer by a space, as seen in thin sections (Nemhauser *et al.*, 1980). These observations suggest that, at least prior to activation, the MB of both *Homarus* and *Limulus* blood cells is in contact with a surface-associated or cortical cytoskeletal layer which is continuous throughout the cell. Interaction between the MB and a sub-surface cytoskeletal layer has been proposed as the basis for MB influence on cell shape in non-mammalian vertebrate erythrocytes (Cohen, 1978; Cohen *et al.*, 1982), and may thus apply to the invertebrate clotting cells as well. If such a surface-associated cytoskeletal layer exists, passage through it would be a major step in secretory granule exocytosis in these cells (Ornberg and Reese, 1981).

MB disorganization during cell spreading

As visualized by indirect anti-tubulin immunofluorescence, lobster clotting cell MBs become disorganized during cell spreading and ultimately disappear. Although microtubule depolymerization and repolymerization may play a role, the images obtained give the impression that the MB initially twists and then splays apart. Splaying or stripping off of microtubules from the major MB bundle also appears to occur in human platelets during spreading, as seen in "spiral ring" forms visualized by anti-tubulin binding. At later stages platelet MBs are not recognizable, but microtubules are present in the cytoplasm and in pseudopodia (Debus *et al.*, 1981). Microtubule disappearance in the case of lobster clotting cells *in vitro* may reflect a more complete process of cellular self-destruction which is part of the *in vivo* lobster clotting mechanism.

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INHIBITION OF GAMETE MEMBRANE FUSION IN THE SEA URCHIN BY QUERCETIN

W. R. ECKBERG[1,2] AND M. E. PEROTTI[1,3]

[1]*Department of Zoology, Howard University, Washington DC 20059,* [2]*Marine Biological Laboratory, Woods Hole, MA 02543,* [3]*Institute of Histology and Embryology and Institute of Human Anatomy, University of Milano, Milano, Italy*

ABSTRACT

We have treated sea urchin gametes with quercetin to investigate the effects of this drug on fertilization and egg activation. High concentrations of this drug gradually inhibited sperm motility, but pretreatment of sperm with the drug did not affect their fertility. Treatment of eggs with quercetin at micromolar concentrations completely blocked fertilization. The drug did not block the acrosome reaction of sperm, nor did it affect the binding of the acrosomal filament to the vitelline layer of the egg. However, fertilization was prevented. Inseminated quercetin-treated eggs could be activated by ionophore A23187, but still failed to incorporate sperm. Quercetin blocked fertilization if added after primary gamete binding, but 30 s before the beginning of the cortical reaction in experiments in which eggs were inseminated at low temperature. Drug added 10 s before the beginning of the cortical reaction was ineffective. Electron microscopy confirmed normal primary gamete binding and failure of fertilization. Vitelline layer removal by trypsin did not restore fertilizability to quercetin-treated eggs. Thus quercetin prevented fertilization by blocking gamete membrane fusion. These results indicate that quercetin can be a useful probe for the mechanism of membrane fusion in fertilization.

INTRODUCTION

There are two generally recognized steps in sperm-egg attachment in the sea urchin; primary gamete binding and membrane fusion. The first is the attachment of the surface of the reacted acrosome to the vitelline layer (Summers *et al.*, 1975). This interaction results from a previous interaction between a fucose-sulfate polysaccharide of the egg jelly coat (SeGall and Lennarz, 1979) and a glycoprotein of the sperm surface (Saling *et al.*, 1982; Eckberg and Metz, 1982). After the acrosome reaction, bindin on the acrosomal filament of the sperm (Vacquier and Moy, 1977) and a glycopeptide receptor of the vitelline layer (Glabe and Vacquier, 1978) cement the gametes together.

The binding of the fertilizing sperm to the egg surface results in a rapid depolarization of the egg surface (Jaffe, 1976; Chambers and deArmendi, 1979) and a rapid block to polyspermy (Jaffe, 1976; Schuel and Schuel, 1981). Subsequent intracellular calcium release leads to the cortical reaction and the activation of egg metabolism (Epel, 1982). Sperm incorporation into the egg is due to the action of microfilaments in egg microvilli. These form the fertilization cone which surrounds the sperm and actively engulfs it (Longo, 1978; Schatten and Schatten, 1980).

We have recently shown that quercetin, an ATPase inhibitor, can initiate meiosis in *Chaetopterus* oocytes (Eckberg and Carroll, 1982). Since meiosis initiation is

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Abbreviation: ASW, MBL formula artificial sea water.

believed to be dependent upon a transient calcium flux within the oocyte (Moreau *et al.*, 1978), we concluded that quercetin might act to initiate meiosis by increasing the intracellular free calcium concentration as the result of inhibition of calcium sequestration. Since such an increase activates sea urchin eggs, we wished to determine whether quercetin might activate sea urchin eggs even though the drug was not found to be parthenogenetic in *Chaetopterus*. Quercetin did not activate sea urchin eggs. Instead, the drug blocked fertilization by sperm, but not activation by ionophore A23187. The results indicate that the drug acts to prevent gamete membrane fusion. A preliminary account of this work has appeared in abstract form (Eckberg and Perotti, 1982).

Materials and Methods

Gametes of the sea urchins *Arbacia punctulata* (Marine Resources Division, Marine Biological Laboratory, Woods Hole, MA), *Lytechinus pictus* and *Strongylocentrotus purpuratus* (Pacific BioMarine Laboratories, Venice, CA) were used in these experiments. Most of the experiments were performed on at least two of the species with quantitatively similar results. *Arbacia* gametes were obtained by electrical stimulation; *Lytechinus* and *Strongylocentrotus* gametes were obtained by KCl (0.5 *M*) injection. Experiments on *Arbacia* and *Lytechinus* were performed at 22–23°C; those on *Strongylocentrotus* were performed at 14–18°C. MBL formula artificial sea water adjusted to pH 8.2 with 10 m*M* Tris-HCl (ASW) was used throughout. Egg vitelline layers were disrupted by trypsin digestion (Saling *et al.*, 1982).

Stock solutions of quercetin (10 m*M*) and ionophore A23187 (1 mg/ml) in ethanol were added to eggs as described (Eckberg and Carroll, 1982). Sperm were added after incubation of eggs in quercetin for 5 min, except where noted. Ethanol had no effect on the phenomena observed at the concentrations tested. To assay for the loss of sperm motility in quercetin, concentrated semen was suspended in ASW to 0.1% (v/v). Various concentrations of quercetin were added and the sperm suspensions were examined at intervals for motility as described (Saling *et al.*, 1982).

To determine sperm binding to eggs in quercetin, eggs were fixed (Eckberg, 1981) at intervals after insemination or A23187 activation and examined by phase-contrast microscopy. For electron microscopy, eggs were postfixed and embedded (Eckberg, 1981), sectioned with a Reichert OM3 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a Siemens Elmiskop 101 electron microscope.

To assay for sperm incorporation, inseminated eggs were fixed in ethanol/glacial acetic acid (3:1, 3 changes), cleared with 45% acetic acid, stained with aceto-orcein and examined microscopically for incorporated sperm. Since sperm attached to the surface of unfertilized eggs in quercetin interfered with such counts, eggs were treated with ionophore A23187 prior to fixation to initiate the cortical reaction and thus to remove attached but unincorporated sperm. Embedded and sectioned eggs were also examined for incorporated sperm by light and electron microscopy.

Results

Figure 1 shows that micromolar concentrations of quercetin effectively blocked fertilization. Concentrations at or above 30 μM were completely effective, while those at or below 3 μM had no effect.

Although quercetin was routinely added to egg suspensions 5 min prior to insemination, Figure 2 shows that the drug exerted its effects on fertilization very

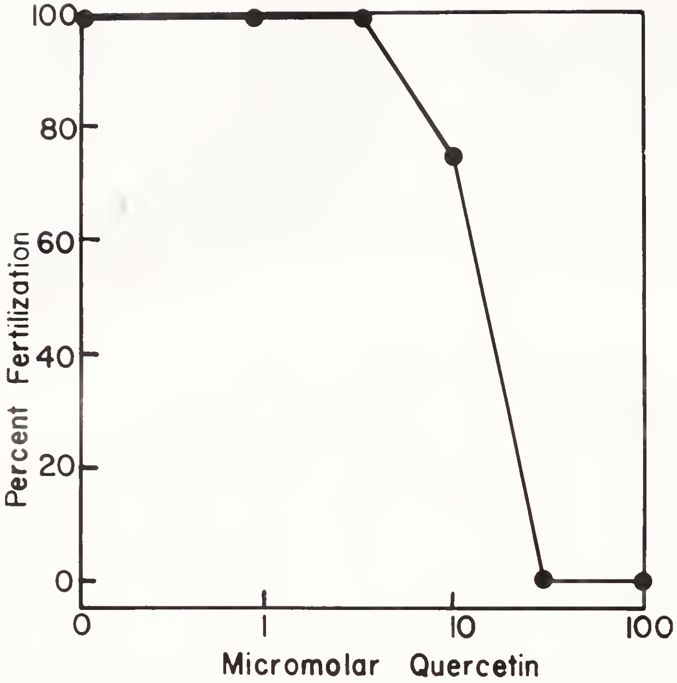


FIGURE 1. Effect of quercetin on fertilization of *Lytechinus pictus* eggs. Eggs were mixed with various concentrations of quercetin in ASW and inseminated five min later. Eggs were examined by phase-contrast microscopy and scored as fertilized if they showed elevated fertilization envelopes, post-fertilization nuclear changes and cleavage.

quickly. Quercetin significantly inhibited fertilization in *Strongylocentrotus* at 14°C when added as late as 20 s after insemination, whereas additions 40 s after insemination were ineffective. Eggs which became fertilized in quercetin subsequently cleaved, indicating that the drug did not directly affect either sperm incorporation, pronuclear migration and fusion, or cell division.

Quercetin treatment was reversible. Sperm or eggs pretreated with 100 μM quercetin and washed with ASW were fertile. Furthermore, inseminated eggs in quercetin could become activated if the quercetin was subsequently diluted with ASW.

Figure 3 shows that quercetin inhibited sperm motility. At fertilization-inhibiting concentrations, sperm motility was reduced within 3 min and ceased with an I_{50} (time of 50% inhibition) of *ca.* 10 min. Lower concentrations of quercetin had no significant effect on sperm motility.

Light and electron microscope analyses showed that sperm could undergo the acrosome reaction in quercetin and bind to and probably penetrate egg vitelline layers but could not activate eggs. Control *Lytechinus* eggs fixed 30 s after insemination had begun to undergo the cortical reaction, but still had numerous sperm bound (Fig. 4). Quercetin-treated eggs fixed at the same time after insemination showed bound sperm but no evidence of egg activation (Fig. 5). Electron microscopy confirmed the fact that egg activation had not begun since all cortical granules were intact (Fig. 6). Electron microscopy also showed that sperm bound to the eggs were attached by the tips of their acrosomal filaments (Fig. 7), and that quercetin had interfered with neither the acrosome reaction nor vitelline layer attachment (primary gamete binding).

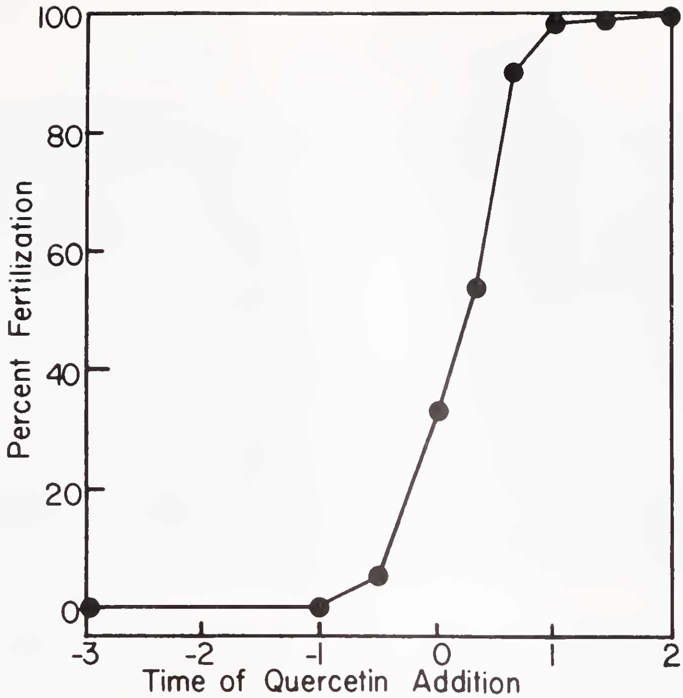


FIGURE 2. Effect on time of quercetin addition relative to time of insemination on egg activation in *Strongylocentrotus purpuratus*. Sperm were added at "0" and quercetin (100 μM) was added at the times indicated relative to sperm addition. Times are given in minutes. Fertilization was determined microscopically as described in the legend to Figure 1.

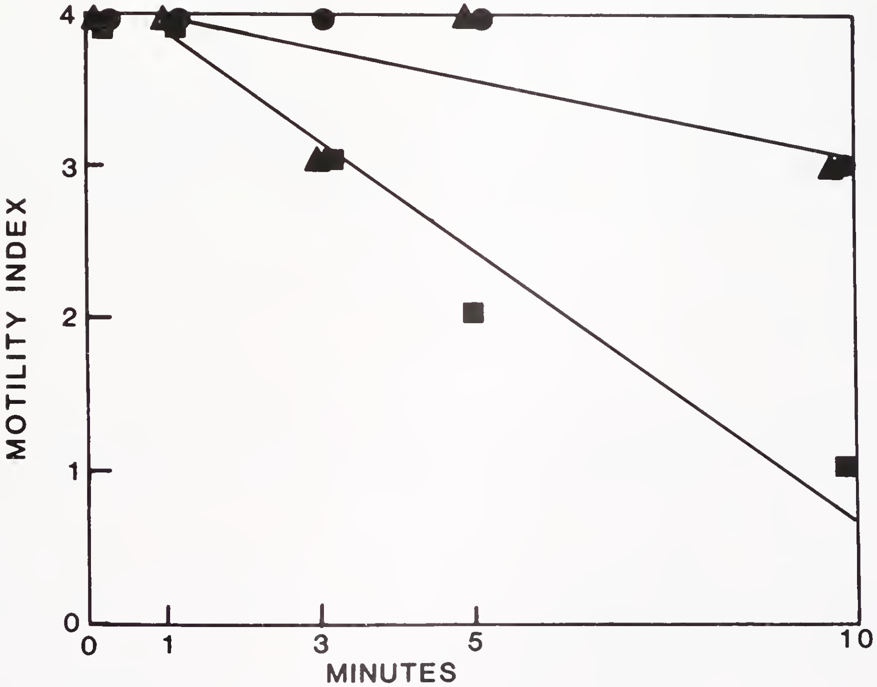
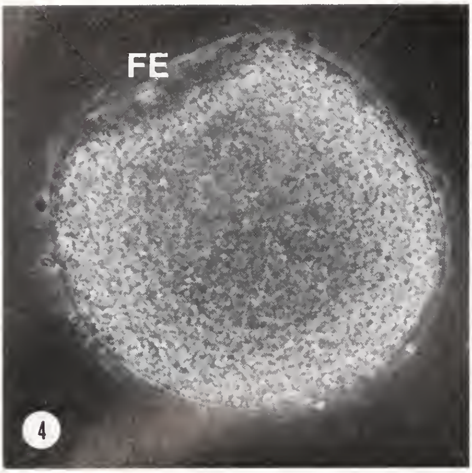
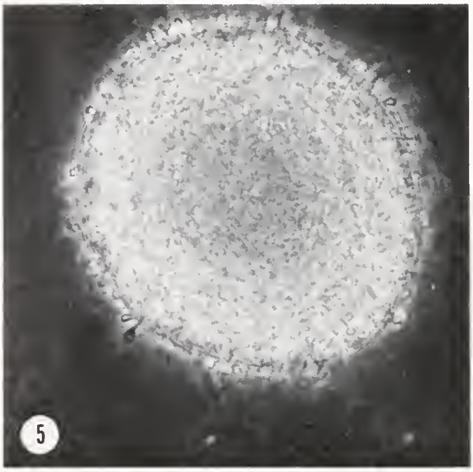
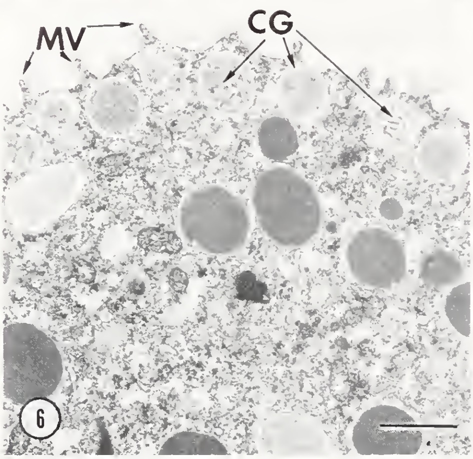


FIGURE 3. Effect of quercetin on the decay of motility by *Lytechinus pictus* sperm. Sperm were incubated either in the absence of quercetin (circles) or in the presence of 10 μM (triangles) or 100 μM (squares) quercetin. Sperm were examined at intervals for motility by phase-contrast microscopy.







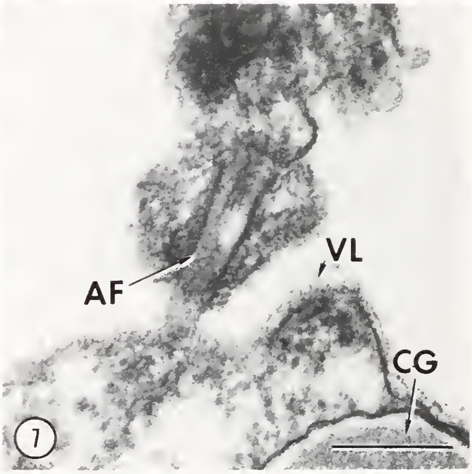


FIGURE 4. *Lytechinus pictus* egg 30 s after insemination in ASW. Note the beginning of fertilization envelope (FE) elevation. Also note that sperm are attached wherever the fertilization envelope has not yet begun to elevate.

FIGURE 5. *Lytechinus pictus* egg 30 s after insemination in 30 μM quercetin. Many sperm are attached to the vitelline layer of the egg, but there is no evidence of egg activation.

FIGURE 6. Surface of a quercetin-treated *Lytechinus pictus* egg. The surface shows typical microvilli (MV) and the cortical granules (CG) are intact. Bar = 1.0 μm.

FIGURE 7. Electron micrograph of a *Lytechinus pictus* sperm bound to the surface of an egg 30 s after insemination in 30 μM quercetin. The acrosomal filament (AF) has attached to the vitelline layer (VL) but the acrosomal process is still surrounded by a continuous membrane. An intact cortical granule (CG) can be seen adjacent to the attached sperm. Bar = 0.2 μm.

If vitelline layer penetration were prevented by quercetin, disruption of the vitelline layer should restore fertilizability. However, trypsin disruption of the vitelline layer did not restore fertilizability (Table I).

By 5 min after insemination, control eggs showed normal fertilization envelopes (Fig. 8), where quercetin-treated eggs still showed attached sperm but no evidence of activation (Figs. 9, 10). Subsequent treatment with ionophore A23187 resulted in fertilization envelope elevation and sperm detachment (Fig. 11). Electron mi-

TABLE I

Effect of quercetin on fertilization of vitelline layerless eggs of Arbacia punctulata.

Vitelline layer	Quercetin	Percent fertilized
present	none	100
present	100 μM	0
absent	none	97
absent	100 μM	0

croscopy confirmed that all cortical granules in these eggs had discharged (Fig. 12). Such eggs were examined for evidence of sperm penetration by light microscopy of cleared whole mounts and sectioned embedded material and by electron microscopy. In no case did we observe any evidence of sperm penetration into the eggs.

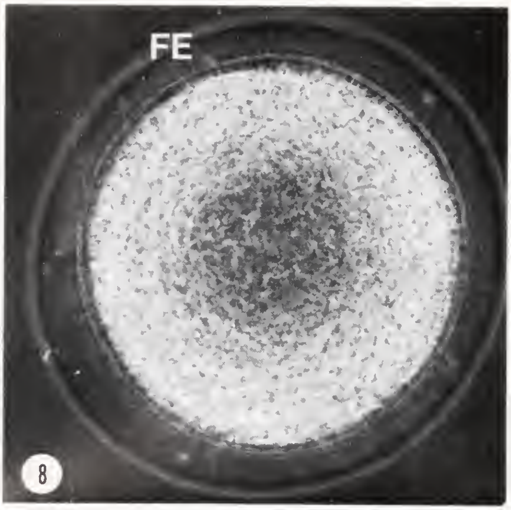
DISCUSSION

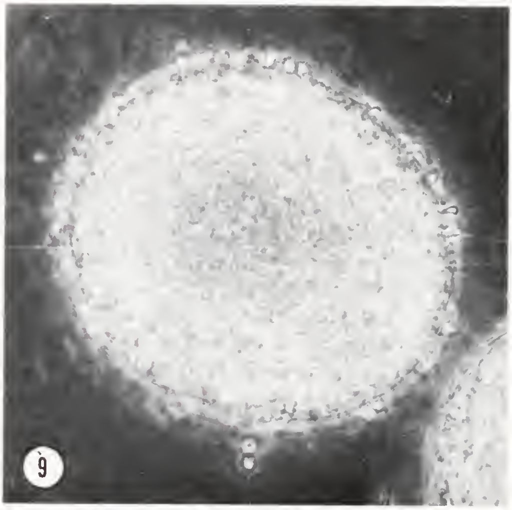
Treatment of eggs with 30–100 μM quercetin blocked fertilization without significantly affecting sperm-egg binding. Although quercetin inhibited sperm motility, this was evidently not the reason for the inhibition of fertilization. Sperm motility decayed only after 1–2 min, after the time at which the fertilizing sperm would have activated the egg. Furthermore, preincubation of either sperm or eggs with quercetin followed by dilution or washing did not affect fertilization. Finally, sperm attached to eggs in quercetin, and such attachment was morphologically normal by both light and electron microscopy. Removal or disruption of the vitelline layer did not restore fertilizability to quercetin-treated eggs. Nor was the failure of egg activation due to direct blockage of the cortical reaction, since ionophore-activated eggs could undergo a normal cortical reaction even after prolonged treatment with at least 200 μM quercetin. The lack of a direct effect on the cortical reaction was surprising since quercetin is known to inhibit secretion in other cells (Fewtrell and Gomperts, 1977). This may indicate that cortical granule exocytosis is partially controlled by a different mechanism than secretion in other cells.

Finally, quercetin did not directly block sperm incorporation. Sperm incorporation is dependent upon microfilaments present in the fertilization cone which forms subsequent to egg activation (Longo, 1978; Schatten and Schatten, 1980). Eggs treated with quercetin after membrane fusion were fertilized, formed asters and pronuclei, and cleaved normally. Quercetin, therefore, had no direct effect on the sperm incorporation apparatus.

Inhibition of fertilization without effects on events up to primary gamete binding and vitelline layer penetration or subsequent to egg activation suggests that quercetin must have blocked some step in the interval between these events. The kinetics of quercetin inhibition of fertilization further indicated that the drug blocked a step at this time. Addition of quercetin to *Strongylocentrotus* eggs up to 30 s before the cortical reaction of control eggs resulted in significant inhibition of fertilization. However, addition of the drug 10-15 s before the cortical reaction was relatively ineffective. Since membrane fusion occurs in this interval and is the only known sperm-egg interaction which cannot be excluded as the affected step, this step must have been blocked.

The mechanism of action of quercetin in inhibiting gamete membrane fusion is not yet clear. Nor is the mechanism of gamete membrane fusion itself known. However, quercetin is known to inhibit membrane enzymes (Fewtrell and Gomperts,


FE
8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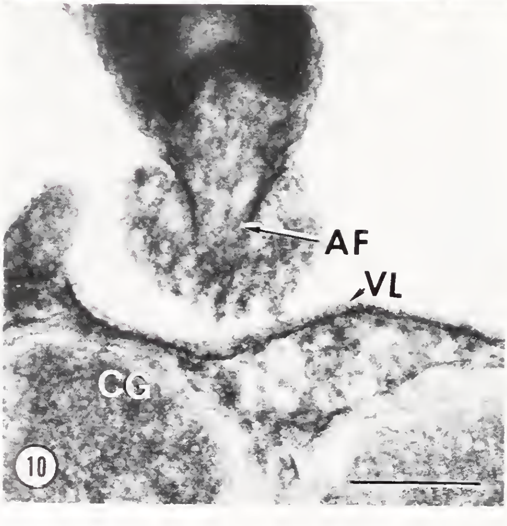
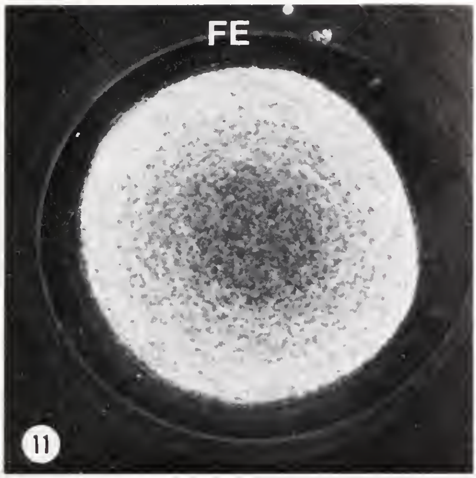

AF
VL
CG
10



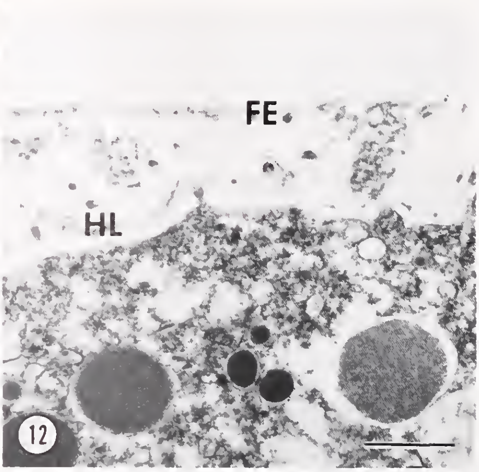


FIGURE 11. *Lytechinus pictus* egg which was exposed to 4μg/ml ionophore A23187. The egg had been treated with 30 μM quercetin for 5 min, inseminated in quercetin and treated with ionophore 5 min later. A fertilization envelope (FE) has formed and sperm have detached.

FIGURE 12. Surface of an ionophore-activated, quercetin-treated egg of *Lytechinus pictus*. Note the presence of a fertilization envelope (FE) and forming hyaline layer (HL) and the absence of cortical granules. Bar = 1.0 μm.

1977), and thus it seems likely that the drug acts by altering the activity or organization of membrane proteins.

The effects of quercetin on gamete fusion may be general. We have recently found that quercetin blocks fertilization in *Spisula* (Eckberg, 1982) and *Chaetopterus* (unpublished data of W. R. Eckberg, L. D. Brown and R. Pettaway). Quercetin probably acts by inhibition of membrane fusion in these species as well.

Fluorescein dyes have recently been suggested to inhibit membrane fusion in sea urchins (Carroll and Levitan, 1978a; Finkel *et al.*, 1981) and in other forms as well (Carroll and Levitan, 1978b). Effectiveness of these anionic dyes was directly proportional to their octanol-water partition coefficients.

The mechanism of action of such dyes that inhibits membrane fusion is unknown, and there is some indication that their action is different from that of quercetin. Erythrosin B but not quercetin decreased primary gamete binding and inhibited egg activation by ionophore A23187 (Carroll and Levitan, 1978a). However, both drugs blocked fertilization when added as late as 20 s after insemination, and both were still effective when egg vitelline layers were disrupted, so they probably both affected gamete membrane fusion. Since quercetin did not affect primary gamete binding or ionophore activation of eggs, quercetin would seem to be a superior drug for inhibition of membrane fusion.

FIGURE 8. *Lytechinus pictus* egg 5 min after insemination in ASW. The fertilization envelope (FE) has completely formed and supernumerary sperm have detached.

FIGURE 9. *Lytechinus pictus* egg 5 min after insemination in 30 μM quercetin. Sperm are still attached and there is no evidence of egg activation.

FIGURE 10. Electron micrograph of a *Lytechinus pictus* sperm bound to the surface of an egg 5 min after insemination in 30 μM quercetin. There has been no change in the mode of sperm-egg attachment. The sperm is still attached by its acrosomal filament (AF) to the vitelline Layer (VL) and the cortical granules (CG) remain intact. Bar = 0.2 μm.

The results of this study indicate that quercetin can be a useful probe for the subcellular components and biochemical processes involved in gamete membrane fusion and fertilization.

ACKNOWLEDGMENTS

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THE CHEMICAL SYSTEMATICS OF COLONIAL MARINE ANIMALS: AN ESTIMATED PHYLOGENY OF THE ORDER GORGONACEA BASED ON TERPENOID CHARACTERS

DONALD J. GERHART

Department of Ecology and Evolution State University of New York at Stony Brook, Stony Brook, NY 11794

ABSTRACT

The classification of colonial marine invertebrates is often very difficult. The purpose of this paper is to illustrate the potential of numerical chemotaxonomy and cladistic analysis to facilitate the study of the evolution of these organisms. Chemotaxonomy has contributed little to the study of these animals, in spite of the large amounts of data generated by marine natural products chemistry. Existing chemosystematic investigations of colonial animals have not employed the methods of numerical taxonomy or of cladistic analysis. As an example of the usefulness of such an approach, cladistic analysis on quantified chemical data was performed for nineteen species of gorgonians. This produced cladograms which, for the most part, agreed with classical gorgonian systematics, but which also yielded several new insights into the evolution and classification of gorgonians. Numerical chemotaxonomy and cladistic analysis should not continue to be ignored as a tool for the study of phylogenetic relationships among groups of colonial marine invertebrates.

INTRODUCTION

The classification of colonial marine invertebrates such as the Porifera (sponges) and the Alcyonaria (octocorals) is often problematical (deLaubenfels, 1936: Bayer, 1961; Wiedenmayer, 1977). These organisms usually lack a well-defined, consistent form. Classifications are based on morphological characters such as color, general growth pattern, polyp morphology (in the Alcyonaria), and particularly the arrangement, color, size, and shape of spicules (Bayer, 1961; Wiedenmayer, 1977). The genetic basis of these characters is unknown, and the environment can exert significant influences on their expression (Bayer, 1961; Grigg, 1970; Kinzie, 1973; Simpson, 1978). Furthermore, the fossil record of most colonial marine invertebrates is incomplete. These problems make it difficult to establish genealogical affinities among the living representatives of these groups.

Chemotaxonomy—the construction of classifications using chemical compounds as characters—could provide new insights into the systematics of colonial marine animals. Both the Porifera and the Alcyonaria possess a diverse array of chemical compounds and are particularly rich in isoprenoids, including terpenes, carotenoids, and steroids (Fenical, 1978 and 1982; Goad, 1978; Tursch *et al.*, 1978; Bergquist, *et al.*, 1980; Liaaen-Jensen *et al.*, 1982). (For a discussion of the possible ecological roles of these compounds in coelenterates, see Tursch *et al.*, 1978). Each species of sponge and octocoral seems to have its own specific set of compounds (Ciereszko and Karns, 1973: Kashman *et al.*, 1980). Marine natural products chemistry has progressed to the point where it is often easier to identify compounds from

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colonial marine organisms than it is to identify the animal from which the compounds were isolated (Faulkner, 1977). In spite of this, chemotaxonomy has contributed little to the study of colonial marine animals. A few chemotaxonomic studies of sponges have been performed (see, for example, Bergquist *et al.*, 1980), but no such studies have been undertaken with octocorals. Existing chemotaxonomic studies of colonial marine invertebrates have not employed the methods of numerical taxonomy, even though chemical characters can be exactly described (Holger, 1968) and thus lend themselves to numerical analysis.

As noted earlier, establishing evolutionary affinities among groups of colonial marine invertebrates is difficult. Cladistic analysis is a technique which allows one to infer phylogenetic relationships from the characters of extant species (Farris *et al.*, 1970; Farris, 1973). A combination of numerical cladistic analysis and chemotaxonomy should greatly facilitate the study of phylogenetic relationships among groups of colonial marine invertebrates. As an example of the application and potential value of such an approach, I performed cladistic analysis on quantified chemical data for nineteen species of gorgonians. Hopefully, this paper will illustrate the immense potential of numerical chemotaxonomy and cladistic analysis for improving our understanding of the evolution of colonial marine invertebrates.

MATERIALS AND METHODS

Tursch *et al.* (1978) described the absolute configuration and species distribution of twenty-eight terpenoid compounds isolated from nineteen species of gorgonians. This data set was used in the present study because the chemical structures presented in it had previously appeared in the literature and were regarded by Tursch *et al.* to be accurate. This information was arranged into a 19 × 28 matrix of ones and zeros, with columns of the matrix corresponding to chemical compounds and rows corresponding to gorgonian species. An entry of "1" in a row and column indicated that the species possessed that particular compound, while a "0" denoted the absence of the compound (see Table I). Cladograms were generated from this matrix by computer using the method of Farris *et al.* (1970).

The carbon skeletons of the compounds were then examined and placed into seventeen biosynthetic categories, based on the pathways of terpenoid biosynthesis outlined by Herout (1971) and Fenical (1978). The bicyclic compounds eunicellin and briarein A were not classified as cembranoid diterpenes. These compounds may arise via unusual cyclizations of geranylgeraniol pyrophosphate instead of a further cyclization of the 14-membered ring of a cembranoid precursor [Fenical, 1978]. Two alcyonacean genera, *Clavularia* and *Cespitularia*, produce tricyclic or bicyclic compounds apparently via an unusual cyclization of geranylgeraniol pyrophosphate [Coll, 1980]. To my knowledge, experimental data on the biosynthesis of eunicellin and briarein A do not exist. The classification of these compounds as non-cembranoids does not drastically alter the position of *Eunicella stricta* or *Briareum asbestinum* in the cladograms.) The presence or absence of a particular class of compounds was noted for each of the 19 gorgonian species. This information was arranged into a 19 × 17 matrix of ones and zeros analogous to the presence-absence data matrix (see Table II), and then generated cladograms from the matrix.

Finally, the presence-absence data matrix was combined with the biosynthetic data matrix to produce a 19 × 45 matrix of ones and zeros. This matrix was also used to generate a series of cladograms.

All cladograms were rooted by adding an all-absent ancestor, a hypothetical ancestor which lacked all terpenoid compounds.

TABLE I

Presence-absence data matrix

	(+)-α-Bisabolene	(+)-β-Bisabolene	(−)-α-Curcumene	(+)-β-Curcumene	(−)-β-Curcumene	(−)-Germacrene	(−)-δ-Cadinene	(+)-α-Muurolene	(+)-β-Gorgonene	(+)-γ-Maaliene	(−)-1(10)-Aristolene	(+)-9-Aristolene	(+)-α-Copaene	(+)-β-Copaene	(+)-α-Cubebene	(+)-β-Epibourbonene	Alloaromdendrene	Furoventlene	Asperdiol	Crassin acetate	Eupalmerin acetate	Eunicin	Jeunicin	Peunicin	Cueunicin	Cueunicin acetate	Eunicellin	Briarein A
Briareum asbestinum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Eunicea asperula	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Eunicea mammosa	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0
Eunicea palmeri	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0
Eunicea succinea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
E. succinea var. *plantaginea*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
Eunicea tourneforti	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Eunicella stricta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Gorgonia ventalina	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Muricea elongata	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plexaurella dichotoma	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plexaurella fusifera	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plexaurella grisea	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plexaurella nutans	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudoplexaura crucis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Pseudoplexaura flagellosa	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0
Pseudoplexaura porosa	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0
Pseudoplexaura wagenaari	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0	0
Pseudopterogorgia americana	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table II

Biosynthetic data matrix

	Farnesanes	Bisabolanes	Germacranes	Bicyclogermacranes	Maalianes	Gorgonanes	Aristolanes	Cadinanes	Copaanes	Cubebanes	Bourbonanes	Aromadendranes	Furoventalanes	Geranylgeranyls	β-cembranoids	Briareins	Eunicellins
Briareum asbestinum	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
Eunicea asperula	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Eunicea mammosa	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Eunicea palmeri	1	0	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0
Eunicea succinea	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
E. succinea var *plantaginea*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Eunicea tourneforti	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Eunicella stricta	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Gorgonia ventalina	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Muricea elongata	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plexaurella dichotoma	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plexaurella fusifera	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plexaurella grisea	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plexaurella nutans	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudoplexaura crucis	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Pseudoplexaura flagellosa	1	0	1	1	0	0	0	1	1	1	1	1	0	1	1	0	0
Pseudoplexaura porosa	1	0	1	1	0	0	0	1	1	1	1	1	0	1	1	0	0
Pseudoplexaura wagenaari	1	0	1	1	0	0	0	1	1	1	1	1	0	1	1	0	0
Pseudopterogorgia americana	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0

RESULTS

The most parsimonious cladograms generated from the presence-absence data matrix, from the biosynthetic data matrix, and from the combined data matrix were selected. This was accomplished by choosing from each series of cladograms, the cladogram with the shortest total length, the lowest F-ratio, and the highest index of consistency. This corresponds to selecting the cladogram which postulated the smallest amount of parallel evolution. (The total length, the F-ratio, and the index of consistency all provide some information on the number of evolutionary reversals contained in a cladogram. For definitions of these measures and for further discussion of their interpretation, see Kluge and Farris [1969], Farris [1972], and Prager and Wilson [1978].) All of these measures suggest that the chemotaxonomic data used here are highly consistent; that is, the amount of parallel evolution which must be postulated to explain the data, was fairly low. The selected cladograms corresponding to the presence-absence data matrix, the biosynthetic data matrix, and the combined data matrix are shown in Figures 1, 2, and 3, respectively. Each line connecting two nodes on the cladogram, or connecting a node with a gorgonian species, is known as a "branch." The nodes of the cladogram denote hypothetical ancestors. The numbers on the branches are known as "branch lengths," which in this case are simply the number of characters not common to both points connected by the branch.

All three cladograms agree remarkably well with Bayer's (1961) classification

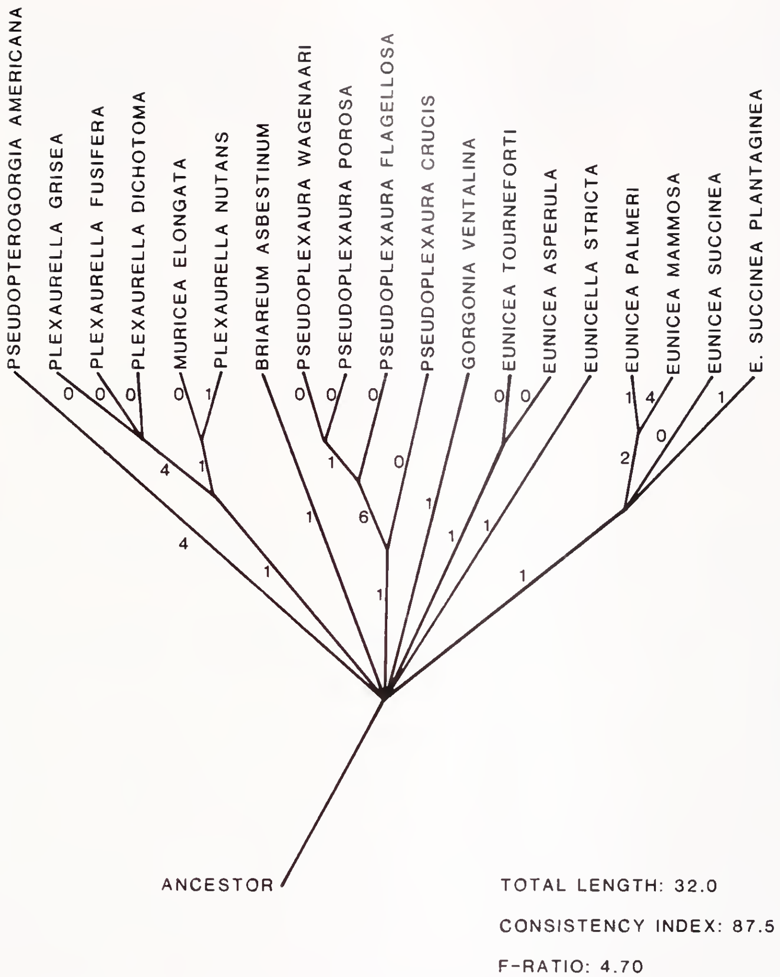
PSEUDOPTEROGORGIA AMERICANA
PLEXAURELLA GRISEA
PLEXAURELLA FUSIFERA
PLEXAURELLA DICHOTOMA
MURICEA ELONGATA
PLEXAURELLA NUTANS
BRIAREUM ASBESTINUM
PSEUDOPLEXAURA WAGENAARI
PSEUDOPLEXAURA POROSA
PSEUDOPLEXAURA FLAGELLOSA
PSEUDOPLEXAURA CRUCIS
GORGONIA VENTALINA
EUNICEA TOURNEFORTI
EUNICEA ASPERULA
EUNICELLA STRICTA
EUNICEA PALMERI
EUNICEA MAMMOSA
EUNICEA SUCCINEA
E. SUCCINEA PLANTAGINEA
0
0
0
0
1
0
0
0
0
0
1
4
1
0
4
1
1
0
2
1
1
6
1
1
4
1
1
1
ANCESTOR
TOTAL LENGTH: 32.0
CONSISTENCY INDEX: 87.5
F-RATIO: 4.70

FIGURE 1. Cladogram 1, generated from the presence-absence data matrix.

of gorgonians (Table III). In all cladograms, congeneric species tend to group together. One cannot usually infer intergeneric relationships from the presence-absence data ("Cladogram 1"). Such inferences can be made in the cladograms generated from the biosynthetic data matrix and the combined data matrix ("Cladogram 2" and "Cladogram 3," respectively), indicating that the addition of biosynthetic data provides a great deal of information on the relatedness of genera.

In Cladograms 2 and 3, *Briareum asbestinum*, which is placed by Bayer (1961) and Kukenthal (1924) in the suborder Scleraxonia (Table III), does not group closely with species of the other gorgonian suborder, the Holaxonia. Furthermore, the genera *Pseudopterogorgia* and *Gorgonia* tend to be separated from the other gorgonian species. Bayer (1961) places these genera in a separate family, the Gorgoniidae.

Eunicella stricta possesses the unusual terpenoid eunicellin and hence shows no close affinities with other gorgonian species in Cladograms 1, 2, or 3. This genus is also peculiar in morphology (even though it is classically placed in the family Plexauridae), having an unusual growth form and possessing "balloon-club" spicules

TABLE III

Classification of the genera examined in this paper, using the taxonomy of Bayer (1961)

- Order Gorgonacea
  - Suborder Scleraxonia
    - Family Briareidae
    - Genus *Briareum*
- Suborder Holaxonia
    - Family Plexauridae
      - Genus *Plexaurella*
      - Genus *Pseudoplexaura*
      - Genus *Eunicea*
      - Genus *Eunicella*
      - Genus *Muricea*
    - Family Gorgoniidae
      - Genus *Gorgonia*
      - Genus *Pseudopterogorgia*

which are unique to this genus (Bayer, 1961). Thus, both chemical and morphological evidence suggest that *Eunicella stricta* is markedly different from the other species used in this study.

The terpenoid data suggest that the genus *Eunicea* should be split into two groups, one containing *Eunicea asperula* and *E. tourneforti*, and the other containing *E. mammosa*, *E. palmeri*, *E, succinea*, and *E. succinea forma plantaginea*. This split corresponds exactly to two subgenera, the subgenus *Eunicea sensu strictu* and the subgenus *Euniceopsis*, proposed by Bayer (1961) on the basis of anthocodial spiculation. Bayer (1961) stated that "future work may demonstrate that these subgenera are actually of full generic importance." Examination of Cladogram 3 shows that the patristic distances (the sum of lengths of branches connecting two taxa—see Farris, 1967) between *Eunicea tourneforti* (or *E. asperula*) and members of the "*Euniceopsis*" subgroup range from 2 to 10, with a mean distance of 6. This suggests that *E. tourneforti* and *E. asperula* are no more closely related chemically to the "*Euniceopsis*" species than they are to species of other genera. Thus, the chemical data considered here support the idea that *Euniceopsis* and *Eunicea sensu stricto* should be elevated to the level of genera.

Cladogram 2 and Cladogram 3 suggest that the order Gorgonacea is comprised of two major evolutionary clades (Fig. 2 and 3). One clade contains the genera *Gorgonia*, *Pseudopterogorgia*, *Plexaurella*, and *Muricea*, all of which possess sesquiterpenes but lack diterpenes. The second clade consists of the genera *Briareum*, *Eunicella*, *Eunicea*, and *Pseudoplexaura*, which all contain diterpenes. Within this latter clade, *Eunicea mammosa*, *E. palmeri*, *Pseudoplexaura flagellosa*, *P. porosa*, and *P. wagenaari* form a sub-clade of species with sesquiterpenes as well as diterpenes. The species of the sub-clade seem to have evolved independently the ability to synthesize large quantities of sesquiterpenes. This implies that the family Plexauridae is diphyletic (see Fig. 3).

There are three groups of chemically indistinguishable species. The first group consists of *Plexaurella fusifera*, *P. dichotoma*, and *P. grisea*; the second contains *Eunicea tourneforti* and *E. asperula*; and the third includes *Pseudoplexaura porosa* and *P. wagenaari* (Fig. 3). Bayer (1961) noted that until further study delineated the range of variability of octocoral species, "the taxonomy of the octocorals will remain in confusion, cluttered with superfluous 'species' that are mere variants of one." The chemical data considered here suggest that each of the three groups should

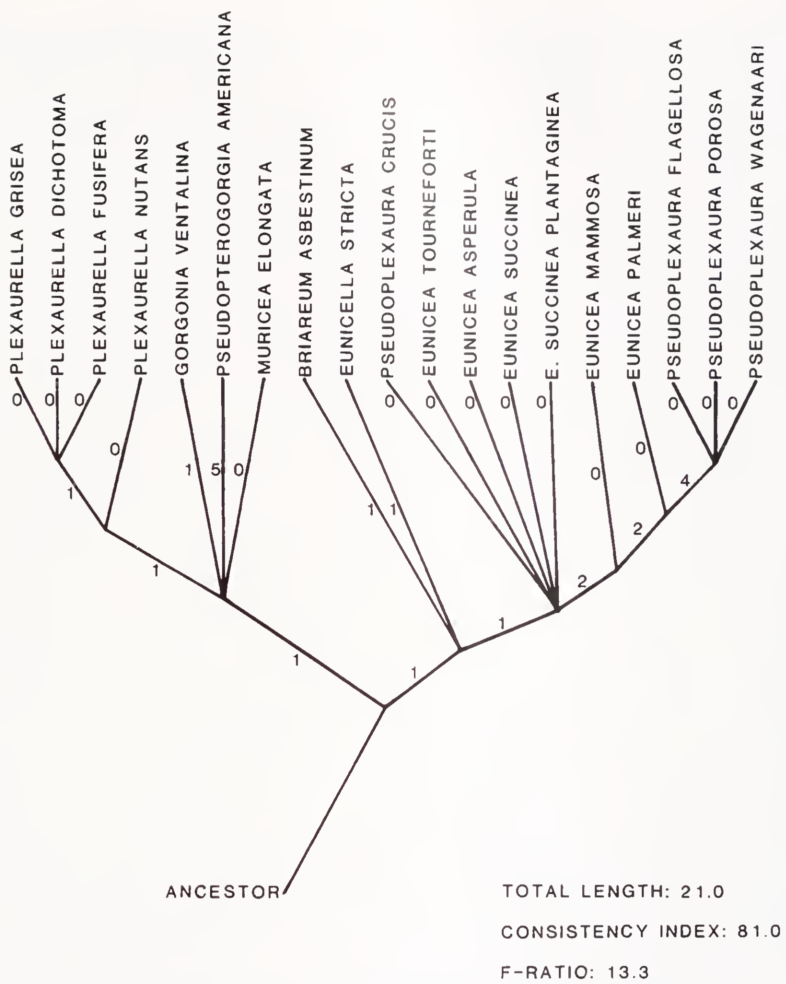
PLEXAURELLA GRISEA
PLEXAURELLA DICHOTOMA
PLEXAURELLA FUSIFERA
PLEXAURELLA NUTANS
GORGONIA VENTALINA
PSEUDOPTEROGORGIA AMERICANA
MURICEA ELONGATA
BRIAREUM ASBESTINUM
EUNICELLA STRICTA
PSEUDOPLEXAURA CRUCIS
EUNICEA TOURNEFORTI
EUNICEA ASPERULA
EUNICEA SUCCINEA
E. SUCCINEA PLANTAGINEA
EUNICEA MAMMOSA
EUNICEA PALMERI
PSEUDOPLEXAURA FLAGELLOSA
PSEUDOPLEXAURA POROSA
PSEUDOPLEXAURA WAGENAARI
ANCESTOR
TOTAL LENGTH: 21.0
CONSISTENCY INDEX: 81.0
F-RATIO: 13.3

FIGURE 2. Cladogram 2, generated from the biosynthetic data matrix.

be consolidated into three species, each containing several variants which correspond to the classical "species." The possibility exists that these species have become reproductively isolated without any divergence occuring in their chemical content. These species should be examined more carefully to ascertain whether they are true species or whether they are simply variants.

DISCUSSION

In general, the chemically based cladograms agree very closely with classical gorgonian taxonomy. Frequently found, for a wide variety of organisms, is a close fit between classifications based on biochemical data and those based on morphological information (Ferguson, 1980). Further work on the biochemical taxonomy of gorgonians will probably provide a great deal of support for Bayer's (1961) classification, while providing several new insights into gorgonian systematics and evolution.

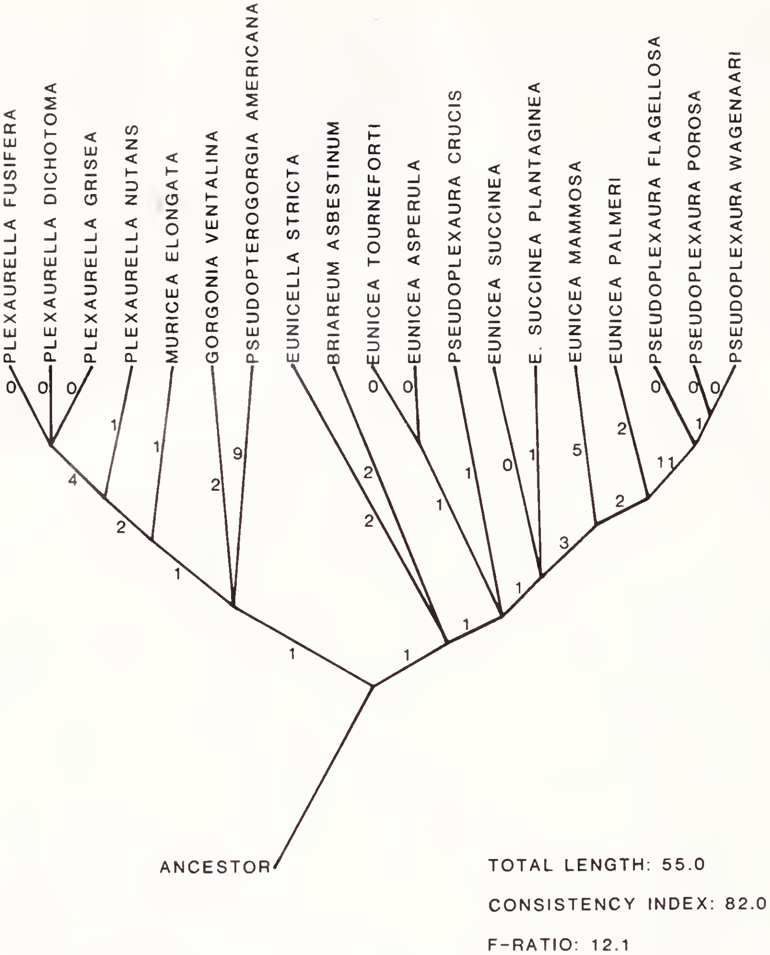
PLEXAURELLA FUSIFERA
PLEXAURELLA DICHOTOMA
PLEXAURELLA GRISEA
PLEXAURELLA NUTANS
MURICEA ELONGATA
GORGONIA VENTALINA
PSEUDOPTEROGORGIA AMERICANA
EUNICELLA STRICTA
BRIAREUM ASBESTINUM
EUNICEA TOURNEFORTI
EUNICEA ASPERULA
PSEUDOPLEXAURA CRUCIS
EUNICEA SUCCINEA
E. SUCCINEA PLANTAGINEA
EUNICEA MAMMOSA
EUNICEA PALMERI
PSEUDOPLEXAURA FLAGELLOSA
PSEUDOPLEXAURA POROSA
PSEUDOPLEXAURA WAGENAARI
0
0
0
1
4
2
1
1
2
9
1
2
2
1
0
0
1
1
0
1
1
1
5
3
2
2
0
1
1
0
0
1
ANCESTOR
TOTAL LENGTH: 55.0
CONSISTENCY INDEX: 82.0
F-RATIO: 12.1

FIGURE 3. Cladogram 3, generated from the combined data matrix.

The classical systematics of gorgonians and other colonial marine invertebrates is based heavily on morphological characters (Kukenthal, 1924; De Laubenfels, 1936; Bayer, 1961; Wiedenmayer, 1977). For gorgonians, spicule morphology and the general growth form of the colony are the characters most frequently used for classification (Bayer, 1961; Grigg, 1970). The degree to which variation in these characters reflects genetic differences between colonies is unknown (Bayer, 1961). However, spicule shape and size can vary considerably within a single gorgonian colony (Bayer, 1961). Several studies have shown that the growth form of a gorgonian can be greatly affected by currents (Wainwright and Dillon, 1969; Grigg, 1970; Kinzie, 1973). Grigg (1970) showed that there was considerable geographic variation in spicule and colony morphology within the gorgonian species *Muricea californica* and *M. fruticosa*. (The variation was so great that Grigg [1970] suggested that they might be different variants of a single species.) When Kinzie (1973) transplanted colonies of *Eunicea clavigera* and *Gorgonia mariae* from deep water to shallow water, he found that spicules near the growing tips of the colonies began to exhibit abnormal characters; furthermore, the spicules of transplanted *G. mariae* changed color from white to red. Simpson (1978) demonstrated that the sponge *Microciona prolifera* produces thicker spicules at lower temperatures. Thus, the environment may have considerable effects on the morphology of gorgonians and perhaps other colonial marine invertebrates. As Bayer (1961) noted, gorgonian taxonomy will remain confused until the normal range of variation of systematically important characters and the effects of the environment upon them have been thoroughly documented.

At present we also know little about the geographic variation of terpenoids in gorgonians. Systematic studies of spatial or temporal variation of terpenoid content of gorgonians have not been undertaken, nor have the environmental effects on chemical content been clarified. In their review of terpenoids in coelenterates, Tursch *et al.* (1978) noted that the gorgonians *Pseudoplexaura porosa*, *P. wagenaari*, and *P. flagellosa* collected from a broad geographic area were very similar in their terpenoid content, while *Eunicea mammosa* yielded the diterpenes cueunicin, cueunicin acetate, eunicin, jeunicin, or eupalmerin acetate depending on where the colonies were collected. *E. mammosa* also showed geographic variation in its content of sesquiterpenes (Ciereszko and Karns, 1973). Tursch *et al.* (1978) reported that similar geographic variation occurs in *Eunicea palmeri* and *E. succinea*. Long-term variation of diterpene content through time also seems to exist in some gorgonians. Within one area in Jamaica, *E. mammosa* contained only jeunicin, while colonies collected from Jamaica in later years contained mixtures of both jeunicin and eunicin (Tursch *et al.*, 1978). *Briareum asbestinum* from one collection yielded briareins A and B, but a collection of this species in the same location one year later yielded briareins C and D. It is possible that chemical content of colonial marine animals varies with the age or reproductive status of the colony. The amount of lipid in the sponge *Haliclona permollis* varies seasonally, and the fluctuations may be related to the reproductive cycle of the colony (Elvin, 1979). Initial studies of terpenoids in the alcyonacean *Capnella imbricata* suggested that seasonal variation in the chemical content of this species may occur (Tursch *et al.*, 1978). More detailed studies of temporal variation in the terpenoid content of colonial marine animals have not been reported. Interestingly spatial and temporal variation in the chemical content of a colonial coelenterate is due to changes in the chemical functionalization of the terpenoid skeleton, not to changes in the skeleton itself (Tursch *et al.*, 1978). Approaches such as the one employed in this paper, which emphasize the importance of the basic carbon skeleton of a terpenoid rather than the presence of certain functional groups in the compound, should reduce the problems posed by geographic and temporal variation in chemical content.

Thus, biochemical characters are subject to some of the same criticisms as morphologic characters. However, biochemical characters are probably better reflections of the genetic differences between species than are morphological characters such as spicule shape. Terpenoids are fairly direct reflections of the action of certain biosynthetic enzymes, which in turn are reflections of the presence of particular genes in the organism. Spicules, on the other hand, are complex structures of calcium carbonate (or silica in the Demospongiae) and are the endproducts of an intricate network of physical processes, each of which may be influenced directly or indirectly by many genes, and which may perhaps be strongly affected by the physical environment.

The paucity of knowledge of the biosynthesis of marine natural products presents another problem for chemotaxonomy. This study has used a biosynthetic scheme for the sesquiterpenes (Herout, 1971) which may or may not represent the pathways operating in gorgonians. (Since use of these biosynthetic pathways leads to cladograms which closely fit classical gorgonian systematics, the scheme of Herout [1971] may actually be valid for the order Gorgonacea.) It may be argued that different species of colonial marine invertebrates could use different biosynthetic pathways to produce the same terpenoid compound. This, however, runs counter to present knowledge of terpenoid biosynthesis in other animals and plants. Clayton (1970) stated that the biosynthetic cyclizations of terpenoids "in general conform to patterns that can be rationalized in terms of non-enzymatic organic chemistry." Thus, in general, two organisms which produce the same type of terpenoid compound will do so via the same biosynthetic pathway. However, the enzymes which catalyze the reactions of the pathway may not be homologous; *i.e.*, the ability of two species to synthesize the same terpenoid may be due to parallel evolution. Cladistic analysis allows parallelism by assuming that characters are reversible (Kluge and Farris, 1969), but if parallelism is rampant, it can cause great difficulties in estimating evolutionary affinities. The cladograms generated in this study for the most part closely fit the classical hierarchy of gorgonian taxonomy, suggesting that parallel evolution of terpenoids is not rampant, and that terpenoid compounds are indeed good characters to use in establishing a taxonomy. These conclusions are also supported by the high degree of consistency of the chemical data. Detailed knowledge of the biosynthesis of compounds in colonial marine animals could provide a great deal of information on the evolution of these organisms. Additional work must be done before this becomes feasible.

The cladograms generated in this paper are based on a limited amount of published chemical data. They are not meant to be serious challenges to accepted gorgonian taxonomy. However, they do suggest several places where classical systematics may be in error and where further work may be warranted. Numerical chemotaxonomy and cladistics could help to clear up many areas of confusion in the classification of colonial marine invertebrates, especially if chemical data were coupled with morphological information. This study is meant to illustrate the great potential of numerical chemotaxonomy for the study of the systematics and evolution of colonial animals. This tool should not continue to be ignored.

Acknowledgments

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THE ROLE OF THE EYE AND CNS COMPONENTS IN PHOTOTAXIS OF THE ARROW WORM, *SAGITTA CRASSA* TOKIOKA

TAICHIRO GOTO AND MASAO YOSHIDA[1]

Department of Physiology, School of Medicine, Gifu University, Tsukasa-cho, Gifu and
[1]Ushimado Marine Laboratory, Kashino, Ushimado, Okayama 701-43, Japan

ABSTRACT

The arrow worm *Sagitta crassa* swims by a slow tactic behavior toward a light of moderate intensities and by a quick target-aiming upon sudden reduction in light intensity. The swimming stroke is achieved by a dolphin-type thrust. Microcautery of eyes, brain and ventral ganglion has shown that one-eyed worms can show the two kinds of photic responses and that specimens with both eyes or the brain damaged can survive, but fail to respond to light. Swimming itself, however, depends on the ventral ganglion being intact. These results indicate that the responses are mediated telophototactically through the eyes, and that no extraocular photoreceptors can be substituted for them. The eye structure which possesses one central pigment cell with seven depressions on four sides is fitted for the telophototaxis, enabling photoreceptive endings in the depressions to be stimulated to different degrees by light coming from one particular direction.

INTRODUCTION

Little is known about the sensory physiology of chaetognaths despite their great importance in marine zooplankton. To date, most research has come from morphological observations of the nervous system and sense organs (Hesse, 1902; Burfield, 1927; Kuhl, 1929; John, 1931, 1933; Eakin and Westfall, 1964; Horridge and Boulton, 1967; Bone and Pulsford, 1978; Ducret, 1978). Some physiological correlations are known in a few cases; the worms respond to moderate amplitudes of water borne vibration (Horridge and Boulton, 1967; Newbury, 1972; Feigenbaum and Reeve, 1977), touch (Bieri, 1966; Fraser, 1969) and light (Esterly, 1919; Spooner, 1933; Pearre, 1973; Goto and Yoshida, 1981). However, information on the mechanisms that link receptor and effector organs is scanty, and direct evidence for the role of the presumed receptors is usually lacking.

In a previous paper (Goto and Yoshida, 1981), two types of light responses were reported for the arrow worm *Sagitta crassa*: a) a slow tactic movement (ST) toward a light of moderate intensities effected by repeated hopping and sinking and b) a quick target-aiming (QTA) by which worms swam straight toward a light source either when the light beam was suddenly reduced or when a water borne vibration was applied. Both of these behavioral responses were thought to be telophototaxes. The present work demonstrates that such responses are executed through the eyes alone. Furthermore, information pathways from photoreceptors to effectors were investigated by cauterizing the eye, the brain and the ventral ganglion in various combinations.

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Send offprint requests to: T. Goto, Ushimado Marine Laboratory, Kashino, Ushimado, Okayama 701-43, Japan.

MATERIALS AND METHODS

Arrow worms, *Sagitta crassa* Tokioka, collected by towing a plankton net were kept for use in aquaria at 17°C with an LD 12:12 cycle (Goto and Yoshida, 1981).

For selective microcautery, only healthy and actively swimming mature worms were used. Worms anesthetized in eugenol containing sea water (100 ppm) were gently placed on an agar plate. The tissues in question were cauterized instantaneously with a steel needle (electrolytically sharpened to 20 μm tip diameter) attached to a radio frequency microcauterizer (100 MHz, 1200 V). Microcauterized worms were returned to aquaria immediately and left for at least two hours. After experimentation, worms were fixed in 2.5% glutaraldehyde, embedded in epoxy resin (TAAB embedding resin), sectioned transversely and stained with toluidine blue. The extent of the cauterization was determined by light-microscopy.

Moribund or inactive worms were discarded. Behavioral experiments were done during the later half of the L-phase of the above cycle. The slow tactic behavior (ST) and quick-target-aiming (QTA) of the microcauterized worms were examined in a specially designed trough (Fig. 1). The trough, 12 × 12 × 4 cm, had a small compartment in one corner (upper right in the figure), which was separated from the main part by a plastic plate, 4 cm in length. Experiments were done in a dark room at temperature ranging from 15°C to 17°C.

ST's were examined in a sequence from a to c (Fig. 1). An arrow worm (shown by an arrow) was dark-adapted in the small compartment for 10 min (a). The partition sheet was lifted up gently (c) and two white light sources, L_1 and L_2 (each being a 100 V-300 W incandescent bulb) were turned on simultaneously. Each beam was collimated by a lens system and passed through an infrared absorbing filter (Toshiba IRA-25S). The intensity of each beam was measured in front of the trough wall by means of a radiometer (Japan Spectroscopic Co., Ltd. Model RMD-1) and was adjusted to become equal, 5.5 μW/mm^2, by neutral density filters (Toshiba). Worms that reached the wall facing either L_1 or L_2 within 10 min were assumed to have responded positively (pluses in Table II).

QTA's were examined in a sequence from b to c in Figure 1. A worm in the small compartment was illuminated for 10 min (b) with three light beams, L_1, L_2 and L_3, the intensity of L_3 being 70 μW/mm^2. The partition was then lifted gently and L_3 was simultaneously turned off (c). The resulting light intensity reduction (about 86%) was the stimulus to induce QTA. Worms which moved immediately toward either L_1 or L_2 by a series of repeated rapid swimming movements were given two pluses (++). One plus (+) was given to those which became simply more active; swimming for a short distance, changing body orientations, etc. A minus (−) indicates no response in both ST and QTA.

Instantaneous profiles of worms showing ST or QTA response were photographed on a single frame of film by delivering a series of strobe flashes (five per sec with an Olympus electronic flash T32) from above with the camera shutter open. To get a sharp focus, a narrow trough, 3.5 cm long, 1.0 cm wide and 2.5 cm deep, was used with the camera aimed at its long side. ST's were induced by illuminating the long axis of the trough horizontally. To study QTA's, a second light was added on the opposite side of that used for ST. Here, the two light sources used were 100 V-30 W incandescent bulbs which provided, respectively, 6.0 and 55 μW/mm^2 at the front surface of the trough. After illuminating the worm to be tested with the two beams for more than 3 min, QTA was induced by extinguishing the brighter light.

To investigate the ultrastructure of the eyes, worms were fixed at room tem-

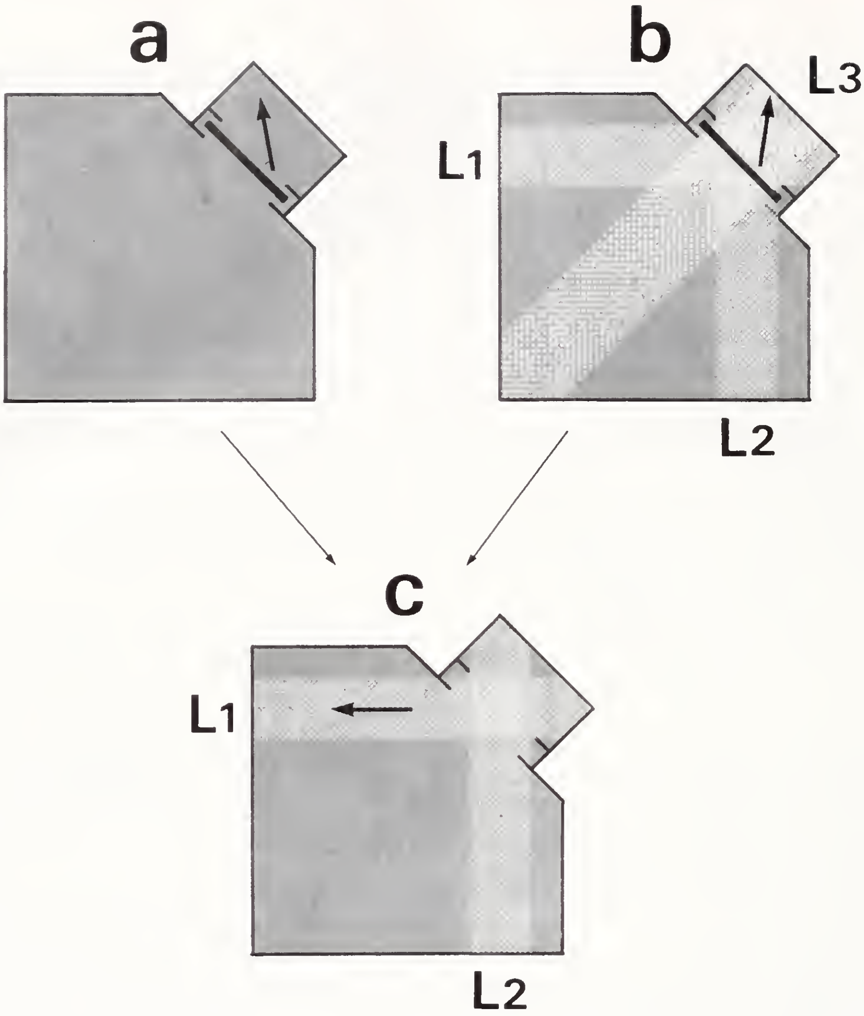

FIGURE 1. Experimental procedure to induce QTA and ST visual response (see text in detail).

perature for 2 h in 2.5% glutaraldehyde and post-fixed for 1 h in 1% osmium tetroxide, both being buffered to pH 7.4 by 0.1 *M* cacodylate containing 0.4 *M* sucrose. After dehydration through a graded series of ethanol, the specimens were embedded in the epoxy resin. Semi-serial EM sections were cut in three planes (transverse, sagittal and horizontal) on a Porter-Blum MT2 ultramicrotome, stained with alcoholic uranyl acetate and lead citrate and examined in a Hitachi H 500H electron microscope.

RESULTS

Orientation of worms responding to light

Sagitta has a pair of large seminal vesicles located laterally to the body axis just anterior to the caudal fin. These appear quite differently when viewed at various

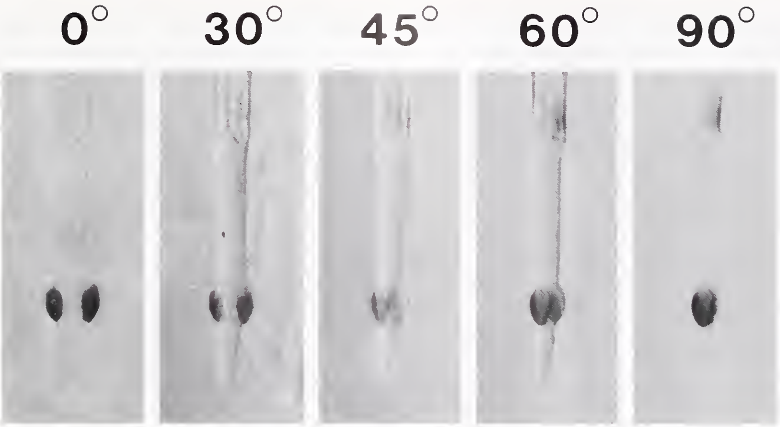

FIGURE 2. Appearance of seminal vesicles viewed from different angles relative to the dorso-ventral axis at 0°. Photographs taken from different angles (0°, 30°, 45°, 60° and 90°) on an agar plate with a narrow and shallow ditch.

angles to the dorso-ventral plane (Fig. 2). Orientations of profiles of swimming worms recorded in strobe flashes can be determined by refering to the appearance of the seminal vesicles. Since the precision of angular inclinations on the pictures was limited, we classified the profiles as dorso-ventral, oblique and lateral orientations, when the seminal vesicles appeared in separation, just attached to each other and overlapped, respectively (Table I).

Four examples are shown in Figure 3 in which a and b are QTA responses. Note that *i*) lateral profiles are seen in the bending phase (both in a and b), *ii*) quickly swimming worms keep the dorso-ventral plane horizontally (profiles on the right in a and one profile on the right in b) and *iii*) the worm in b, which oriented in the opposite direction against the light source, did show the QTA response. When sinking passively, however, the dorso-ventral axis gradually changed from more or less horizontal to vertical. During an ST response lateral and oblique profiles were recorded (Fig. 3c). The abrupt shift in position without a bending profile shown from left three profiles to the middle group in Figure 3c indicates the rapidity of the bending stroke occurring between strobe flashes delivered at 0.2 sec intervals.

The relation of *Sagitta*'s dorso-ventral plane, hence the orientation of eyes, with respect to the light source was studied by taking pictures while the animal was either approaching the light source by ST or was stimulated to swim by QTA. Table I summarizes the results of changes in orientation of the dorso-ventral plane, determined by using two or three successive profiles. Binomial tests were made to reveal whether differences observed between dorso-ventral and lateral orientations in each phase of reaction were significant or not.

Several important implications are evident. First, most of the bending worms (Table I b) were recorded as lateral profiles. Hence the body's motion in swimming strokes apparently occurs dorso-ventrally. In other words, the quick swimming is achieved not by an eel-like lateral stroke but by a dolphin-type thrust. Second, during the gliding phase (Table I c and d) after the first stroke most intact worms orient their dorso-ventral plane horizontally. Although one-eyed worms seem to show the same tendencies in Table I, the differences between dorso-ventral and lateral orientation were not statistically significant. Third, orientation of the dorso-ventral axis was random under all the other conditions. The last result implies that i) regardless

TABLE I

The number of incidences of orientations around the antero-posterior axis when the worms were in varying phases of motions in response to horizontally directed light and in passively sinking phases

The motion of the worm	Worm's eye	Orientations: Dorso-ventral	Oblique	Lateral	Binomial test*
QTA					
a. immediately before bending	intact	6	1	9	NS
	one eye	2	0	0	NS
b. in bending	intact	3	5	26	P < .001
	one eye	0	1	6	P < .05
c. immediately after bending	intact	23	4	7	P < .001
	one eye	5	1	1	NS
d. reached the lit wall	intact	20	3	8	NS
	one eye	7	0	2	NS
ST					
e. immediately before bending	intact	14	9	5	P < .05
	one eye	14	6	11	NS
f. immediately after bending	intact	15	4	9	NS
	one eye	22	5	4	P < .001
control					
g. passively sinking	intact	6	2	11	NS

* Statistical significance of difference between dorso-ventral and lateral orientations.

The orientations recorded by a camera aimed at the long side of a trough were determined by the appearance of seminal vesicles.

of the initial body orientation (Fig. 3a and b, Table I a), the worms can locate a visual target in QTA responses and are correctly aligned within a fraction of a second, ii) in ST responses, more frequent tilting of the antero-posterior body axis toward the light source (Goto and Yoshida, 1981) can be achieved under any orientation of the dorso-ventral plane (Table I f), and iii) when the chaetognaths are sinking passively, their dorso-ventral plane is randomly oriented (Table I g). The results obtained with one-eyed worms approaching the target by ST movements may suggest a preferred orientation of the dorso-ventral plane for the visually defective worms to locate a light source (Table I f).

A few comments may be added as regards the item (i). The data presented here are all concerned with rotation around the long axis of the body. One may ask the importance of the vertical inclination of the long axis. A part of the answer may be found in Figure 3b in which the worm oriented in the opposite direction against the target to go. Re-examination of the data which we accumulated for publication of a single photograph in our previous paper (Fig. 5 in Goto and Yoshida, 1981) revealed that 51 out of 71 worms had oriented in the opposite direction against the target before they responded by QTA's. This result does not necessarily mean that the opposite direction was preferable for the animal to locate that target but was simply due to our experimental procedure (cf. Fig. 7 in Goto and Yoshida, 1981)

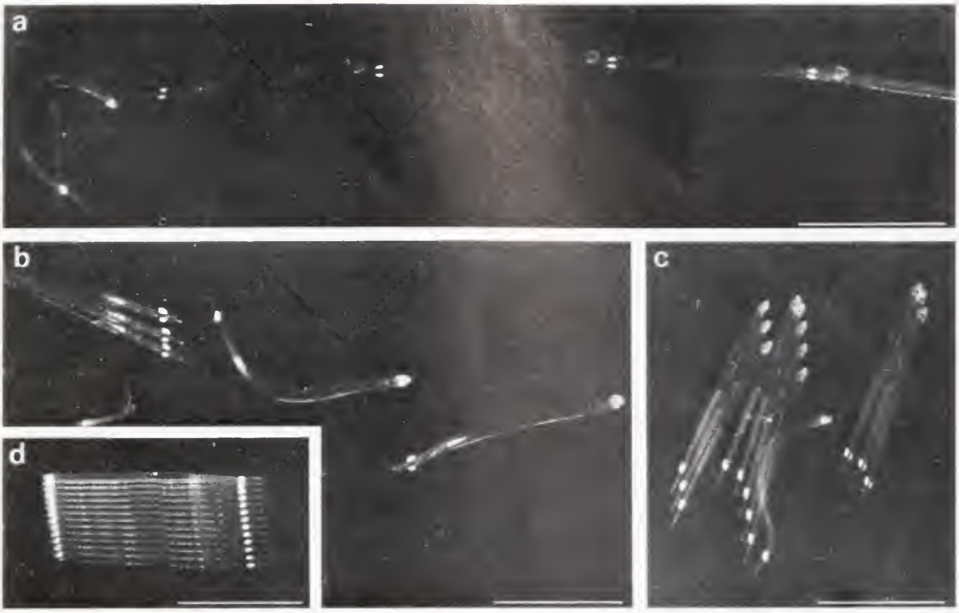

FIGURE 3. Examples of profiles of *Sagitta* showing QTA responses (a and b), ST response (c) and passive sinking (d). The target light source was to the right. Scale bars indicate 0.5 cm. a: The bending profile of stroke to the left is followed by gliding profiles with horizontal dorso-ventral plane. b: Passively sinking worm to the left points to the right with a bending profile in the middle. c: Three shots to the left and five in the middle show lateral profiles and three to the right show oblique ones. d: The dorso-ventral plane is more or less horizontal in the uppermost profile and vertical in the lowest profile.

whereby the animals were accumulated in one end of the trough opposite to the target by their ST movements.

Effect of selective microcauterization

Behavioral results obtained with the worms which were cauterized in varying ways are summarized in Table II and light micrographs of the cauterized eye regions are shown in Figure 4. Nine out of 10 of the intact control worms which recovered from anesthetization (Fig. 4a) showed both types of normal response. One worm failed to show QTA. Hence the eugenol exposure was without significant aftereffect. Sham control experiments were made by cauterizing the epidermis covering the area between the two eyes (Fig. 4b). Though some of the microcauterized worms became unresponsive, more than 60% of the 18 worms concerned responded normally (b in Table II). Considering the delicacy of their body structure, the ability of *Sagitta* to retain responsiveness after local cauterization may be considered high.

Worms in which one eye (d in Fig. 4 and Table II) and some of the epidermis above it (c in Fig. 4 and Table II) were cauterized resembled the sham control worms in their responses. Cauterization at two sites (e in Fig. 4 and Table II) increased the number of unresponsive worms, but only when both eyes were completely destroyed (f in Fig. 4 and Table II) did the microcauterized worms lose all their responsiveness to light. Even so, they could still swim in a disoriented manner.

We next selectively cauterized the brain and the ventral nerve ganglion on the trunk which lie just under the epidermis at some distance anterior and posterior to the eyes, respectively (g and h in Table II). Surprisingly, worms with a damaged

TABLE II

Effects of microcauterization of various tissues upon two types of light-oriented responses of Sagitta crassa

	ST		QTA		
Microcauterized tissue	+	−	++	+	−
a. none (control)	10	0	7	2	1
b. epidermis between two eyes	11	7	12	2	4
c. epidermis above one eye	12	5	8	3	6
d. one eye	13	7	7	4	9
e. epidermis above two eyes	3	6	2	0	7
f. both eyes	0	5	0	0	5
g. brain	0	4	0	0	4
h. ventral ganglion	0	3	0	0	3

Intact worms experienced anesthetization before experiments. One plus for slow-tactic responses (ST); the worm tested reached the wall facing either one of the light sources within 10 min. Two pulses for quick target-aiming (QTA); the worm tested swam rapidly and reached one of the lit walls. One plus (QTA); the worm tested simply became more active. Minus (ST on QTA); "no response". The figures indicate the number of worms which showed the respective type of responses.

brain showed the normal 'hop and sink' swimming pattern, but they did not respond to light even though both eyes were intact. On the other hand, worms with a damaged ventral ganglion but with the brain left intact no longer swam but sank passively down to the bottom.

Eye structure

Chaetognaths are known to possess a pair of lensless eyes under the epidermis (as shown in Fig. 4a) of the head region. Each eye consists of four kinds of cells; capsule cells which surround the external surface of the eye, one centrally located pigment cell surrounded by photoreceptor cells of an inverted ciliary type and glial cells among the photoreceptor units.

It has been held that the pigment cell of each eye in *Sagitta hexaptera* (Hesse, 1902), *S. bipunctata* (Burfield, 1972) and *S. scrippsae* (Eakin and Westfall, 1964) have five depressions. The present data on *S. crassa* reveal (Fig. 5) that the pigment cell at the center has seven depressions on four sides, *e.g.*, one anterior, one posterior, one lateral and four equal medial ones. A simplified model is shown in Figure 5d. Visual cells around the pigment cell extend into its depressions distal endings which presumably comprise the photoreceptive site (Eakin and Westfall, 1964; Ducret, 1978). Light coming from one particular direction will be attenuated largely by pigment granules. Though incasement by depressions of the pigment cell is not complete, the amount of light absorbed by the distal endings will inevitably be different among seven groups of sensory cells. Semi-serial sections revealed that the proximal ends of the sensory cells extended as optic nerve fibers (Fig. 6a) anteriorly to the brain. There were about 90–100 axons in transverse sections of the optic nerve (Fig. 6b) but no synapses were found between the eyes and the brain. This number of axons is rather small compared with *S. scrippsae* which has 500–600 (Eakin and Westfall, 1964).

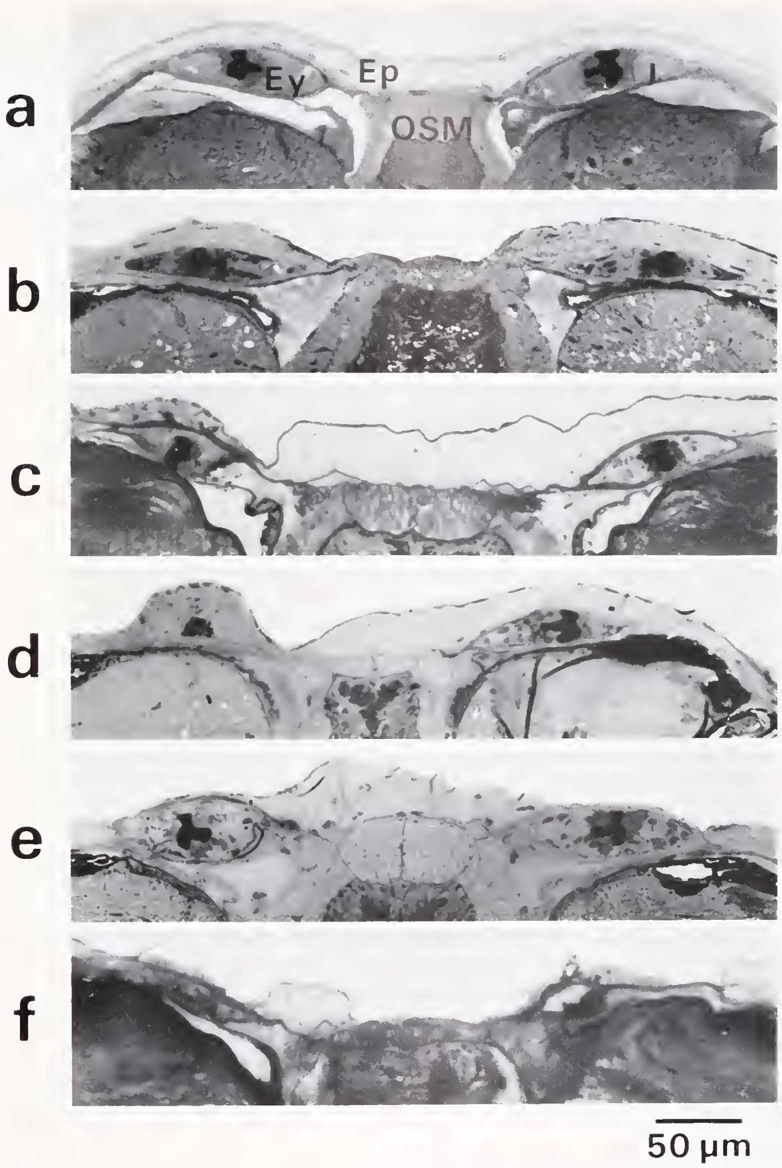

FIGURE 4. Light microscopic profiles of the ocellar region of *Sagitta*. a: Intact. b: Sham control with epidermis injured between the two eyes. c: Epidermis above left eye injured. d: Left eye damaged. e: Epidermis above both eyes injured. f: Both eyes completely damaged. Ep, epidermis; Ey, eye; OSM, oblique superficial muscle.

DISCUSSION

When arrow worms were responding to two identical beams of light either by ST or by QTA movements, none of the worms neither intact nor sham-operated, took an intermediate path between the two beams. Following the terminology de-

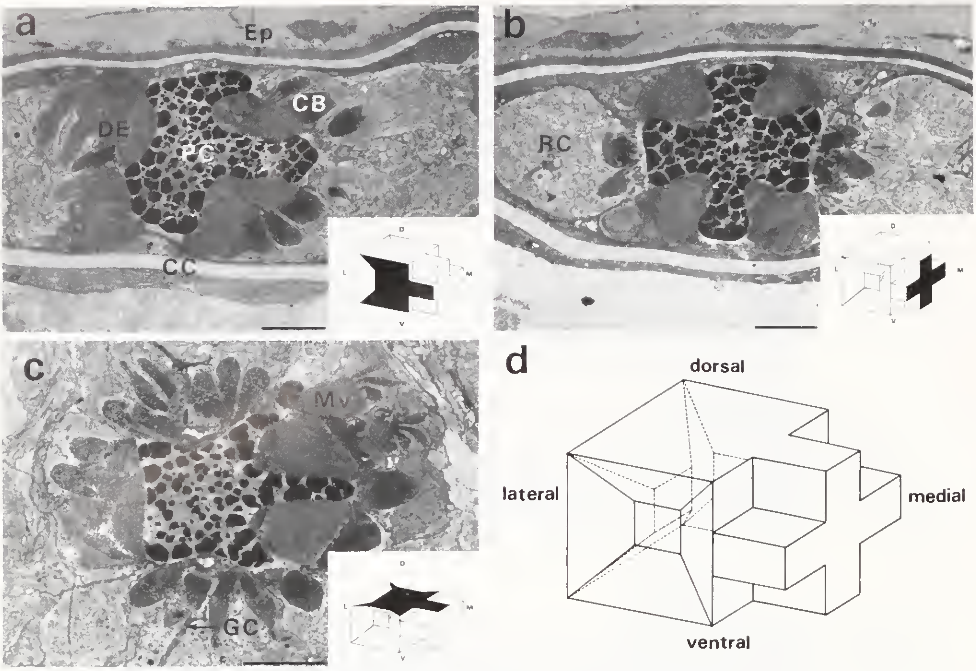

FIGURE 5. Electron micrographs of transverse (a), sagittal (b) and horizontal (c) profiles of one eye and schematic representation of the pigment cell (inserts and d). CB, conical body; CC, capsule cell; DE, distal ending of the photoreceptor cell; Ep, epidermis; GC, glia cell; Mv, microvilli; PC, pigment cell; RC, receptor cell. Scale bars indicate 5 μm.

fined by Frankel and Gunn (1961), both types of photic responses may be referred to as telotaxis since both eyes were not necessary for the oriented responses. Our microcauterization experiments demonstrated that the presence of one eye was sufficient for the worms to swim accurately toward the light source (Table II).

There is no direct physiological evidence that the so-called eyes of arrow worms are in fact photoreceptors, though this seems likely from what is now known to their morphology (Hesse, 1902; Burfield, 1927; Eakin and Westfall, 1964; Ducret, 1978). The present experiments prove that arrow worms lose all responsiveness to light without both eyes. This means that the eyes are the photoreceptive sites for the two light responses studied and that no extraocular photoreceptors can be substituted for them.

When microcauterization injured only the epidermis above the two eyes, some worms still showed light responses even though the light reaching the eye from the normal direction was presumably attenuated largely by the damaged epidermis. This indicates that effective light stimuli for target location can pass through the transparent body from other directions.

Hyman (1959) wrote: "The forward dart is so rapid as to be difficult of analysis but is believed to result from the alternate contractions of the dorsal and ventral longitudinal muscle bands of trunk and tail". The dorso-ventral bendings recorded at the time of swimming strokes clearly show involvement of those muscle bands. When gliding rapidly toward the light source after the first bending stroke, however, worms rotate around their antero-posterior axis by 90° so that the dorso-ventral axis is horizontal. The resulting vertical orientation of two pairs of the lateral fins

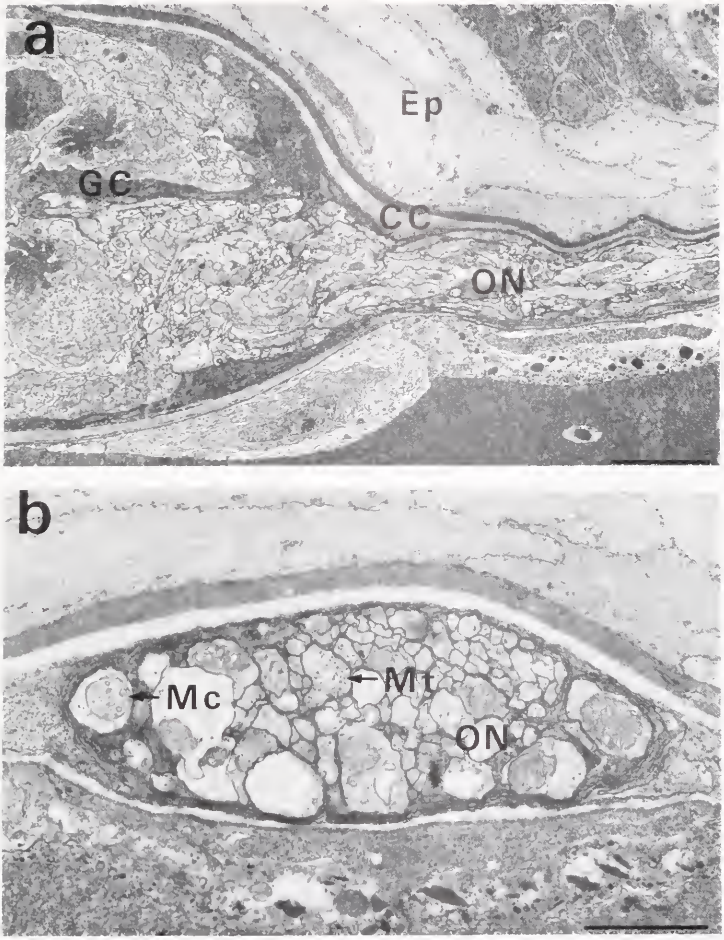

FIGURE 6. Electron micrographs of sagittal (a) and transverse (b) profiles of a bundle of optic nerves. In a, the eye is to the left. CC, capsule cell; Ep, epidermis; GC, glial cell; Mc, mitochondria; Mt, microtubule; ON, optic nerve. Scale bars indicate 5 μm in a and 2 μm in b.

may be advantageous for the worms to keep the target-aiming path during the course of gliding phase.*

The fact that the worm can locate a light source regardless of its orientation either to the dorso-ventral or to the antero-posterior axis can probably be explained by the structure of the eye. As described above, the eye of the arrow worms has photoreceptive endings which are separated into seven groups by the centrally located pigment cell. Due to its shielding effect, a particular group of the endings will receive light coming from a particular direction more effectively than the others. Synapses are lacking between sensory cells and along the optic nerve. A specific combination of excitation pattern which depends on a corresponding direction of the incident beam may be processed in the brain as an information to locate the target. Combined with the results obtained by injuring the brain and the ventral ganglion, the latter may be assumed to be a locomotor center in which responses to light are controlled by the eyes through the brain.

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* We thank one of the referees for this suggestion.

The effect of water borne vibration in inducing QTA movements and sensing of the inclination angles of the antero-posterior axis in the ST response (Goto and Yoshida, 1981) lead one to expect some kind of mechanoreceptors. Cilia distributed over the body surface may be part of such a system (Burfield, 1927; John 1933; Horridge and Boulton, 1967; Bone and Pulsford, 1978). Detailed analyses and hopefully recordings of the ventral ganglion's input and output pathways as well as those of the brain, are needed to improve our understanding of the unique behavioral patterns of chaetognaths.

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THE EFFECTS OF TEMPERATURE AND SALINITY ON LARVAL GROWTH OF THE HORSESHOE CRAB *LIMULUS POLYPHEMUS*

ROY LAUGHLIN

University of California, Naval Biosciences Laboratory, Naval Supply Center, Oakland, CA 94625

ABSTRACT

Horseshoe crab, *Limulus polyphemus*, eggs were reared through the first tailed stage in factorial combinations of temperature and salinity with respective values of 20, 25, 30, or 35°C and 10, 15, 25, or 35‰. Lowest survival occurred in low temperature, low salinity combinations (20°C, 10‰). Wet weight, and thus linear dimension, of the first tailed stage was only slightly different throughout the temperature-salinity range tested. However, ash-free dry weights, indicating yolk utilization, varied significantly with both temperature and salinity. The least yolk utilization occurred at 30°C and increased in higher and especially lower temperatures. Yolk utilization also increased with a decrease in salinity. Temperature interacted with salinity to cause a greater salinity-dependent difference in yolk utilization at lower temperatures. Comparisons of ash-free dry weights (*i.e.*, yolk utilization) with respiration rates indicate that *L. polyphemus* has little ability to compensate metabolically for the effects of temperature and, secondarily, of salinity. Nevertheless, the larvae are sufficiently provisioned with yolk to survive the prevailing ranges of these variables in the habitat where adults normally deposit eggs. This suggests that other unexamined physical variables, or more likely biological factors such as predation or competition among the feeding larval stages and adults, are important for larval survival of *L. polyphemus*.

INTRODUCTION

The brooding behavior of the horseshoe crab, *Limulus polyphemus*, is unique among marine macrofaunal arthropods. A brooding pair in amplexus deposits the eggs in moist sand at or near the water line (Lockwood, 1870; Packard, 1872). Depending primarily upon temperature, the eggs develop for a period of weeks or perhaps months to the trilobite, the first free-living stage (Jegla and Costlow, 1979; 1982). The trilobites generally remain close to the shore, but may also join the plankton (Rudloe, 1979, 1981). The first description of the trilobite stage in the scientific literature was based on material collected from the plankton (see Packard, 1872). In the nearshore environment, both physical and trophic factors are unpredictable. Anecdotal evidence indicates that the animals are very tolerant of suboptimal conditions (Kingsley, 1892), including low salinity (Mangum *et al.*, 1976). Since the trilobite depends on stored yolk material and does not feed, it is protected from trophic instability until after the molt to the first tailed stage.

The ability to tolerate a range of physical factors exacts some cost in terms of metabolic adjustment which must be integrated within the constraints imposed by the amount of energy and material originally supplied within the egg. If development were excessively slowed by low temperatures or osmotic regulation requirements, then growth and perhaps survival would be influenced adversely by these suboptimal

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conditions. The experiments reported here were designed to determine the ranges of temperature and salinity over which development to the first tailed stage would occur. Oxygen consumption rates were measured in order to compare the effects of physical factors on energy production and development rate. Following molting to the first tailed stage, the ash-free dry weights were determined. Differences in ash-free dry weight reflect relative amounts of yolk utilized during development.

MATERIALS AND METHODS

The eggs used in this experiment were collected in June, 1979 near Panacea, Florida (USA) by the Gulf Specimen Company and shipped to California by air. Two clutches showing the least development were selected. Based on the complete lack of morphological differentiation in the eggs and the amount of time required subsequently for development, the eggs were probably less than 1 wk old at the initiation of the experiment, and there was probably approximately a 2 day difference in the age of the two clutches.

During shipment, the eggs were in 25‰ sea water. Previous experience had indicated that the developing embryos were quite tolerant of salinity and temperature changes. On the first day of the experiment the eggs were divided into the experimental groups and put directly into the test temperature-salinity combinations with the exception of the 10‰ groups. These were put into 15‰ for the first 24 h. The embryos were exposed to factorial combinations of temperature (20, 25, 30, or 35°C) and salinity (10, 15, 25, or 35‰) for the remainder of larval development to the first tailed stage.

Oxygen consumption rates were determined for free-living trilobites under all temperature-salinity combinations. Individuals for respirometry were not selected until after all the trilobites emerged from their hatching membranes. Oxygen consumption rates were determined using the oxygen electrode in a Lexington Instruments (Lexington, Massachusetts, USA) blood gas analyzer (Laughlin *et al.*, 1979).

The sea water was changed 5 days each week and the animals in the bowls were censused daily. As necessary, cast egg shells and embryonic membranes were removed and algae cleaned from the bowls using paper tissue. No food was added since none of the stages used in this experiment required it. As the larvae molted to the third tailed stage, they were removed from the bowls, blotted dry, weighed to the nearest 0.01 mg, and dried for at least 2 days at 60°C before being weighed to the nearest 0.1 μg. Subsequently, the tissue was ashed in a kiln at 475°C and the ash weight determined to the nearest 0.1 μg. All weights were measured with Cahn 21 electrobalance.

The figures give means and standard deviations. Two means whose standard deviations do not overlap are different at the 5% level. Data were analyzed statistically using the regression subroutine in the Statistical Package for the Social Sciences, SPSS, (Nie *et al.*, 1975). A regression model containing quadratic and linear terms for temperature and salinity and the linear interaction term was generated and plotted.

RESULTS

Survival to the first stage varied with physical factor combinations. The percent survival is given by the numbers at the base of each histogram column (Fig. 1). Under optimal conditions, more than 50% of the eggs developed to free-living trilobites. In combinations of low temperature (20°C) and low salinity (10 or 15‰) survival was notably poor. There was an unusual pattern of mortality. In several

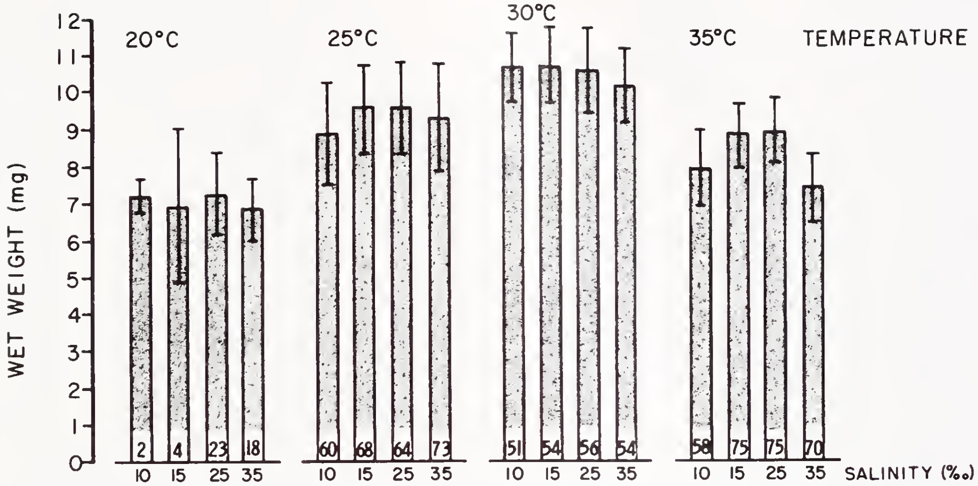

FIGURE 1. Wet weights of first tailed stage *Limulus polyphemus* exposed to factorial combinations of temperature and salinity. The numbers near the base of each column denote the percentage of animals successfully molting to the first tailed stage. Error bars show one standard deviation of the mean. Two hundred eggs were tested in all temperature groups except 35°C where only 40 eggs per salinity were tested.

instances, trilobites survived over 6 wk past the mean time of molting for their group, gradually becoming moribund prior to death. This was especially common at 20°C.

Wet weights varied with both temperature and salinity (Fig. 1). The highest mean wet weights occurred in all salinities at 30°C (~10 mg) and decreased in the order 25° (~9.2), 35°C (~8.5 mg) and 20°C (~7 mg). The effect of salinity was secondary to that of temperature. Within each temperature group, the reductions in wet weight were neither large nor consistent. The largest difference within one temperature (17%) occurred at 35°C, with 25 and 35‰.

Ash-free dry weights, the weights of organic material in the first tailed stage, showed a consistent response to both temperature and salinity at temperatures between 20 and 30°C (Fig. 2). The effect of temperature here was similar to that on wet weights. The largest amount of organic material (~1000 μg per larva) was present in trilobites raised at 30°C. The mean ash free dry weights of larvae at 25 and 35°C were less than at 30°C, with respective values of 900 and 850 μg per larva. The lowest mean weight, 750 μg per larva occurred at 20°C. The effect of salinity on ash-free dry weights was remarkably consistent in all temperatures except 35°C and even here the exceptions were not great. There was a 50–100 μg increase in these weights with a 5–10‰ salinity increase. The relative effect of temperature on the salinity-mediated changes in weight was smallest at 30°C and became progressively larger as the temperature decreased to 20°C. The effect of low salinity on ash-free dry weight is obvious when the differences in mean weights in the 10‰ groups and among the other salinity groups are compared. The 5‰ salinity decrease from 15–10‰ caused a decrease in ash-free dry weight equal to or greater than that caused by a 10‰ change in higher salinities. The growth pattern in 35°C did not show the regular weight increases with salinity which occurred in lower temperatures, and an interaction between temperature and salinity was apparent at 35°C, since the mean ash-free dry weight for the 35‰ salinity group declined approximately 25 μg from that of the 25‰ group.

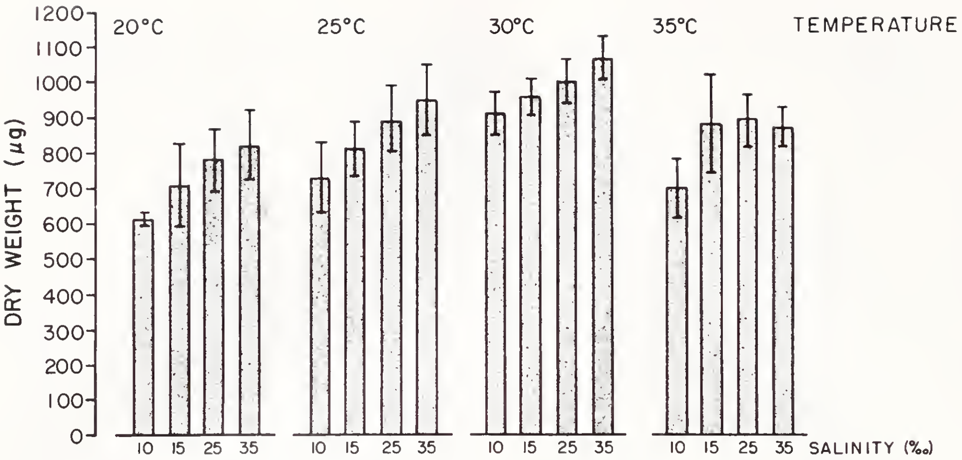

FIGURE 2. Ash-free dry weight of first tailed stage *Limulus polyphemus* exposed to factorial combinations of temperature and salinity. Bars show one standard deviation of the mean.

These data were analyzed by response surface analysis. The following regression equation predicts the effects of temperature and salinity on ash-free dry weight of the trilobites:

$$\text{Ash-free dry weight} = -0.0326 + 0.474T - 0.069T^2 + 0.159S - 0.0084S^2 - 0.021T \times S$$

$$R = 0.614$$
$$F = 174$$
$$df = 4,\ 1155$$
$$p < 0.0001$$

where

T = temperature
S = salinity
R = correlation coefficient
F = F-test ratio
df = degrees of freedom

The response surface shows a ridge of maximum response which is tilted just off and above the 30° isocline (Fig. 3). The ridge descends with decreasing salinity. The effect of low salinity-low temperature combinations is very evident here, with predicted decreases in ash-free dry weights of 45% from a high of 1.00 mg to only 0.55 mg under low temperature-salinity combinations.

Development rate was strongly influenced by temperature (Fig. 4). The shortest mean time required to reach the first tailed stage, 20 days, occurred at 30°C. The 35°C groups required almost a week longer to molt to the first tailed stage. As temperature decreased from 30°C, there was a decrease in development rates and an increase in variability among the groups, indicating an interaction between temperature and salinity. The groups reared in 25°C required between 41 and 51 days to molt, while at 20°C this was dramatically increased to 89–176 days with an even larger range for the different salinity groups. (Some of the trilobites actually survived several more weeks at 20°C before dying without molting).

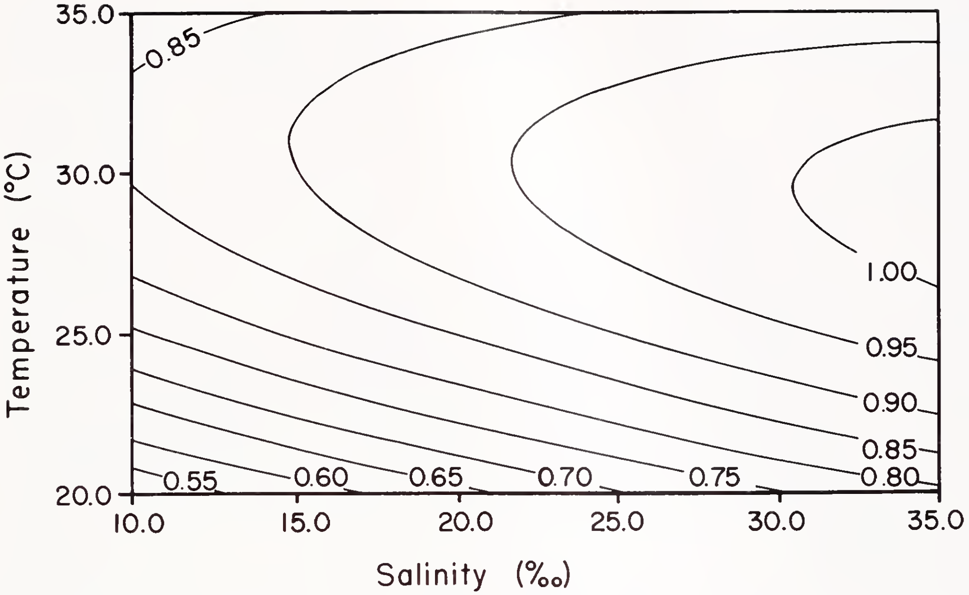

FIGURE 3. Response surface diagram showing predicted ash-free dry weights of first tailed stage *Limulus polyphemus* exposed to factorial combinations of temperature and salinity. Contour units are mg.

Both high (35‰) and low (10‰) salinities had delaying effects on development rate, but the magnitude of the delay was mediated by temperature. At 30°C, the optimum temperature, salinity had little effect on development rates; whereas, at 35°C a slight effect at high and low salinities was apparent. At 25°C development

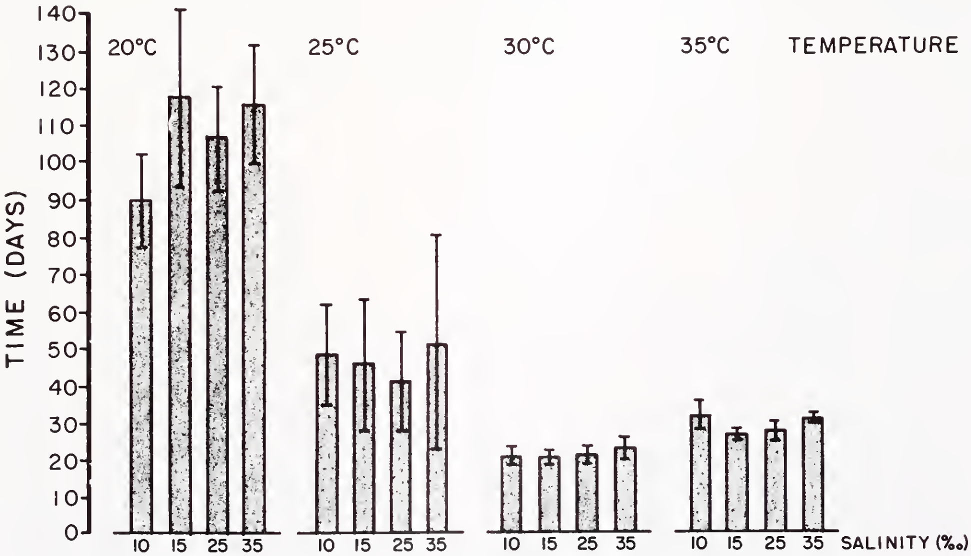

FIGURE 4. Duration of larval development to the first tailed stage of *Limulus polyphemus* exposed to factorial combinations of temperature and salinity. Bars show one standard deviation of the mean.

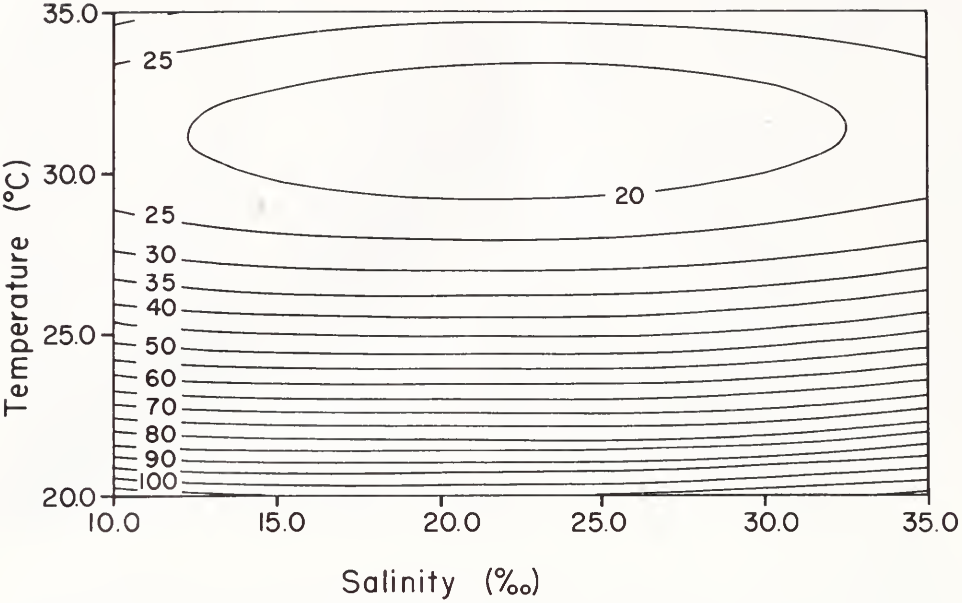

FIGURE 5. Response surface diagram showing effects of temperature and salinity on predicted development rates to the first tailed stage of *Limulus polyphemus*. Contour units are days.

was most rapid in 25‰, decreasing slightly in both higher and lower salinities. The differences were not large, however. At 20°C, temperature was exerting the main effect, expressed as high mortality and very low weights of surviving first tailed stage individuals.

The response surface diagram showing the effects on temperature and salinity (Fig. 5) was plotted from the following regression equation:

$$\text{Days to molt} = 207.2 - 111.6T + 17.5T^2 - 7.2S + 20S^2 - 0.86T \times S$$

$$R = 0.854$$
$$F = 311$$
$$df = 4, 1155$$
$$p < 0.0001$$

where

T = temperature
S = salinity
R = correlation coefficient
F = F-test ratio
df = degrees of freedom

The optimal temperature is predicted to be ~31°C with a broad salinity optimum. The major axis of the central ellipse is nearly parallel to the salinity axis indicating little interaction between temperature and salinity.

Temperature was the major factor influencing oxygen consumption (Fig. 6). The Q_{10}'s were generally less than 2 indicating metabolic compensation. This compensation was least effective in the highest (35‰) and lowest (10‰) salinities tested. As

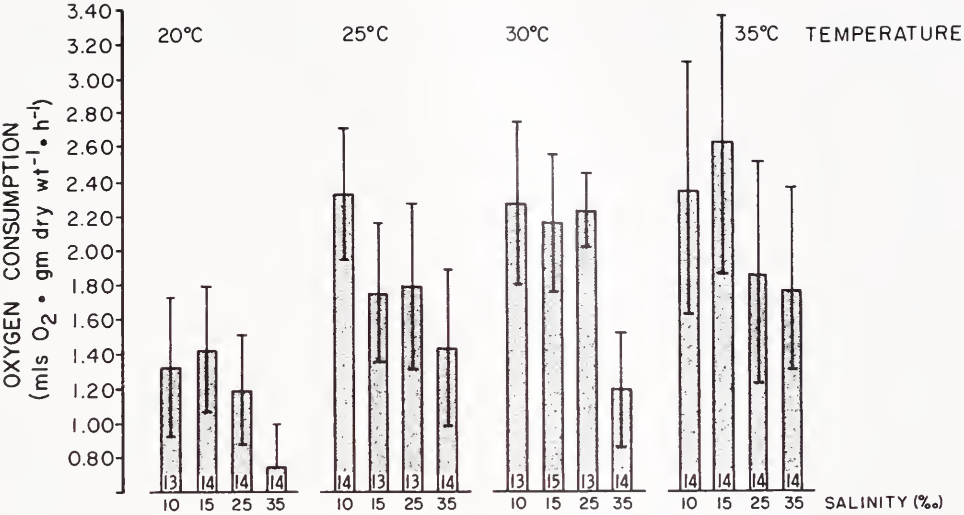

FIGURE 6. Oxygen consumption rates of free-living *Limulus polyphemus* trilobites acclimated to indicated temperature-salinity combinations prior to respirometry. Bars show one standard deviation of the mean. The numbers at the base of each column indicate the number of replicates.

a general trend, the oxygen consumption rates decreased with increasing salinity at each temperature. The relationship of temperature and salinity on oxygen consumption is given by the following equation:

$$\text{Oxygen consumption} = 1.434 + 0.536T - .00083T^2 + .0314S - 0.000745S^2 - 0.0000413T \times S$$

$$R = 0.658$$
$$F = 37.8$$
$$df = 5, 214$$
$$p < 0.001$$

where

T = temperature
S = salinity
R = correlation coefficient
F = F-test ratio
df = degrees of freedom

The temperature, but not the salinity, effect is clearly shown (Fig. 7). The contours are nearly perpendicular to the temperature axis.

DISCUSSION

Although *Limulus polyphemus* developmental stages tolerate a wide range of temperature-salinity combinations, these data indicate that they are comparatively more sensitive to low temperatures than to low salinities, within the normal environmental ranges. Survival is high, at least in some factor combinations, at tem-

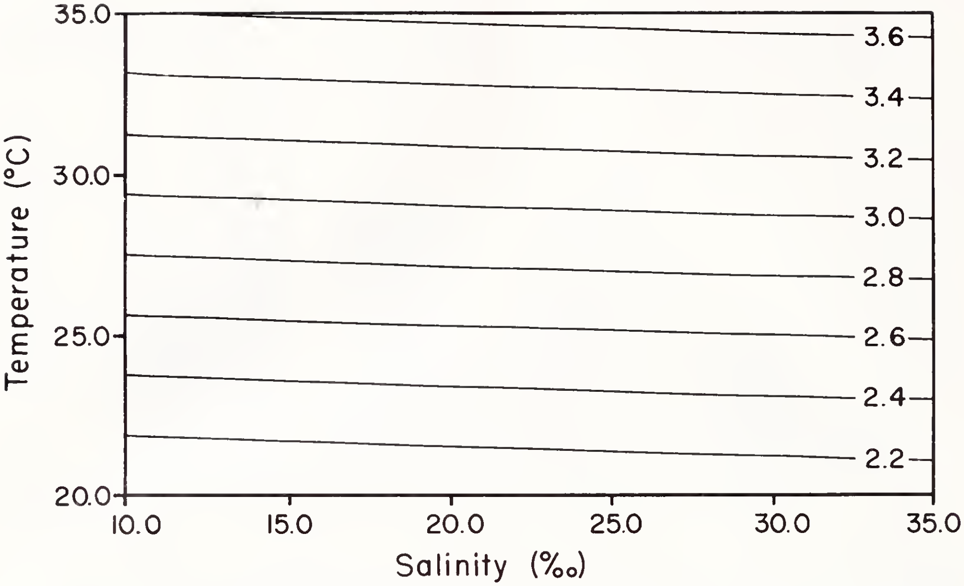

FIGURE 7. Response surface diagram showing predicted oxygen consumption rates of *Limulus polyphemus* acclimated to factorial combinations of temperature and salinity. Contour units are mls $O_2 \cdot$ g dry $wt^{-1} \cdot h^{-1}$.

peratures of 20–35°C and salinities of 10–35‰. Sublethal effects on growth and development rate are marked, however.

Wet weights varied with temperature but not nearly to the same extent as with salinity, and overall the differences were not large. The lack of a clear-cut salinity effect is surprising since one might predict a larger animal in lower salinities due to post-molt swelling. However, the yolk loss in low salinities (Fig. 2) produced an initially "smaller" larva which failed to reach larger proportions regardless of any putative differential swelling. Other explanations are also possible since post-molt water uptake mechanisms are not characterized for *Limulus* and may not be similar to those reported for decapods (Mykels, 1980). Ecologically, attainment of a certain minimum dimension may be an important deterrent to predation since many predators (*e.g.*, fish) are highly size-selective for their prey. Rudloe (1981) found correlations between the size of *Limulus polyphemus* juveniles and their distance from the shore. This observation indicates (but does not prove) that younger, smaller horseshoe crabs in shallow water could avoid predators limited by water depth.

Several observations can be cited to show that temperature effects on yolk utilization are mediated primarily by changes in development rate. Oxygen consumption consistently increased over the temperature range tested; however, development rate was most rapid at 30°C, declining slightly at 35°C and markedly as temperature decreased to 20°C. Dry weight of the third tailed stage animals decreased as the duration of development increased. These observations indicate that even at 20°C oxidative metabolism could provide sufficient energy for developmental events. Therefore, there appears to be only a loose coordination between the rate at which energy is supplied and is used to promote development. Hubbs (1926) suggested that a similar lack of coordination between tissue formation and differentiation is the reason that fish embryos reared in colder temperatures generally have more somites following differentiation events. The situation with *Limulus* represents another paradigm of similar origin. Development rate and oxidative metabolism are best co-ordinated at 30°C, for the population tested, but this decreases in higher or lower temperatures. Rearing at 20°C, the lowest tested here, markedly affected survival, indicating that there is a point of no return. If too much carbon is used for maintenance metabolism before developmental events proceed far enough to allow a molt, the animals cannot molt. This is not a case of simple starvation, however, since the trilobites will live weeks or in some cases months following the mean molting time of successful siblings. The process in *Limulus* may have a similar origin and controlling factor to that studied in starved crab larvae (Anger *et al.*, 1981). While hormonal mechanisms have been suggested, perhaps other mechanisms, at the level of cell metabolism, should be considered since the existence of a point of no return appears to be a salient feature of invertebrate metamorphosis in general (Thorson, 1946, 1966).

The data for *Limulus* show that the effect of temperature on molting, growth, and differentiation is complex. Studies of post-metamorphic fish have provided many experimental models of the effects of temperature on growth, but these are probably inaccurate for *Limulus* or other invertebrate larvae. Alderdice (1972) has reviewed many of the studies on fish and has noted that while growth to a certain size was slower in lower temperatures, growth efficiencies (weight gain per food input) were higher. For *Limulus*, the decelerating effects of low temperature on development rates were more detrimental than any benefit derived from lower maintenance metabolism costs. This is observed for species with feeding as well as non-feeding larvae (Rice and Provenzano, 1965; Provenzano, 1967, 1968; Gore, 1970; Peckenik, 1979; Anger and Dawirs, 1981; Caswell, 1981; Laughlin and Neff, 1981). The occurrence of latitudinal populations of *Limulus*, as well as numerous other species, which develop under very different temperatures shows that temperature sensitivity is under selective control.

There is anecdotal evidence suggesting that juveniles of *Limulus polyphemus* are more salinity tolerant than adults (Mangum *et al.*, 1976), but there is only one study of the mechanisms involved (Laughlin, 1981). The data reported here are for salinities as low as 10‰, but *Limulus* will actually survive and develop to the trilobite stage in salinities as low as 5‰. However, based on the results here, it is doubtful they would survive to the first tailed stage at these low salinities.

Decreasing salinity predictably reduced the ash-free dry weight of the first tailed stage, with the extent of reduction between highest and lowest salinity being mediated secondarily by temperature. Previous studies indicate that loss of carbon is not mediated by costs of ionic regulation. Intracellular osmotic regulation in *Limulus* is mediated by an organic solute which has not been specifically identified (Bricteux-Gregoire *et al.*, 1966). Commitment of organic carbon to osmotic regulation, particularly if it were excreted rather than being "recycled", (for instance by oxidative deamination) in low salinities, could explain the growth pattern with relation to salinity.

The success of *Limulus* larvae, in ecological terms, is dependent upon the choice of breeding beaches where eggs are deposited, as well as the tolerance of larvae to prevailing physical conditions after deposition. I am not aware of any studies specifically addressing the physical factor regime on *Limulus* breeding beaches, but anecdotal evidence and personal experience suggests that adults do not occur where conditions exceed the broad temperature-salinity ranges tolerated by larvae. It is likely that the broad tolerance range of larvae is an adaptation to post-depositional events which adults do not experience. Rain may significantly dilute salinities, and temperatures may increase with season, particularly along the southeastern coast of North America. Thus, the broad temperature and salinity tolerance range of *Limulus* larvae is not an adaptation allowing exploitation of a large range of habitats within an estuarine system, but rather an insurance against unusually large deviations in temperature and salinity which occur randomly in degree and duration from year to year. The apparently copious stored yolk material endows *Limulus polyphemus* larval stages with a resource base allowing survival in the unpredictably variable estuarine habitat.

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THE INFLUENCE OF INORGANIC IONS AND ACCLIMATION SALINITY ON HEMOCYANIN-OXYGEN BINDING IN THE BLUE CRAB *CALLINECTES SAPIDUS*

RICHARD P. MASON[1], CHARLOTTE P. MANGUM[1] AND GERALD GODETTE[2]

[1]*Department of Biology, College of William and Mary, Williamsburg, VA 23185 and* [2]*Marine Biomedical Center, Duke University Marine Laboratory, Beaufort, NC 28516*

ABSTRACT

The effects of salinity changes on the oxygen binding properties of hemocyanin have been examined in the blue crab *Callinectes sapidus.* Oxygen affinity increases measurably with increases in Ca^{+2}, Mg^{+2} or Na^+ but, within the physiological range, not K^+. Unlike its large and specific influence on other arthropod hemocyanins, Cl^- has little or no effect. Ca^{+2}, alone and in physiological concentrations, restores oxygen affinity to the level observed in a complete physiological saline. Ca^{+2} also restores the Bohr shift to the physiological level. Physiological variation in inorganic ions (or pH) does not affect the cooperativity of oxygen binding.

Physiological variation in Ca^{+2} explains only a minor fraction of the actual change in oxygen affinity that accompanies acclimation to a new salinity. The acclimation, which occurs within 8 days, requires a non-dialyzable factor in the blood. The non-dialyzability of the factor and the previous reports of a large increase in blood protein levels at low salinity might implicate an intrinsic change in the hemocyanin molecule with salinity, a suggestion that is also supported by apparent differences in the subunit composition of the molecule in the high and low salinity acclimated crabs. Using paired observations on the same individuals before and after acclimation, however, we were able to demonstrate relatively small changes in hemocyanin concentration. Moreover, the relationship between the differences in subunit composition and HcO_2 binding is uncertain. The identity of the factor remains unknown.

INTRODUCTION

The influence of the ionic environment on the oxygenation properties of the hemocyanins (Hcs) has been recognized for many years. Among the crustaceans, HcO_2 affinity increases with the addition of inorganic ions and decreases with the addition of H^+ (Redfield, 1933; Larimer and Riggs, 1964; Chantler *et al.*, 1973; Truchot, 1975; Mangum and Towle, 1977; Brouwer *et al.*, 1978). In many euryhaline species the opposite responses to pH and inorganic ions are especially important because they actually occur *in vivo*, with the net effect of stabilizing the performance of the HcO_2 transport system (Truchot, 1975; Weiland and Mangum, 1975). A detailed theoretical analysis of the opposite effects of Mg^{+2} and H^+ suggests that the mechanism is competition at the same ion-binding sites, a few of which are linked to O_2 binding sites (Arisaka and Van Holde, 1979).

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Abbreviation: Hc, hemocyanin.

The influence of physiological changes in inorganic ions on other HcO_2 binding properties has not been extensively investigated in the crustaceans; the available information suggests that physiological changes in blood salts have little or no effect on the Bohr shift (Truchot, 1975) or cooperativity (Miller and Van Holde, 1981).

The influences of the various inorganic ions found in the blood are not equal. On a molar basis, divalent cations have a greater effect on HcO_2 affinity than any of the monovalent ions (*e.g.* Truchot, 1975; Brouwer *et al.*, 1978). On a molar basis, however, physiological changes in organic ions are also unequal. Divalent cations are both less abundant and, in most species, regulated far more strongly than Na^+ and Cl^-. Should they prove to be effectors, the monovalent ions could be more important (Mangum, 1981).

Although monovalent cations have not been widely examined, the effects of Cl^- and HcO_2 affinity have been investigated in a number of arthropods, with surprisingly different conclusions. A specific Cl^- effect was first demonstrated in the chelicerate *Limulus* by Sullivan *et al.* (1974) and later elucidated by Brouwer *et al.* (1977). The chelicerate and crustacean hemocyanins have many structural and functional dissimilarities, however. Of greater relevance here, a clear effect of total salinity was found in the brachyuran *Callinectes sapidus* (Mangum and Towle, 1977), which maintains a very nearly homeostatic condition of the divalent cations in its blood (Colvocorresses *et al.*, 1974). The specificity of a quite large Cl^- effect was demonstrated in the dendrobranchiate crustacean *Penaeus setifer* by Brouwer *et al.* (1978). Although the specificity of the individual ions is not yet known, clear effects of both total salinity and mixtures of Cl^- salts have also been found in other crustaceans such as the thalassinid shrimp (Miller and Van Holde, 1981).

In contrast, Truchot (1975) reported that in the brachyuran *Carcinus maenas*, a member of the same family as *C. sapidus*, the effect of NaCl on HcO_2 affinity is very small, non-specific and, at least within the physiological range, opposite to the salt effects observed in other crustaceans. Using constants calculated from curves describing the response to each of several inorganic salts, he concluded that the response to total salinity can be explained almost in full by changes in the divalent cations, which are regulated less perfectly in *C. maenas* than in *C. sapidus*.

With one exception (Miller and Van Holde, 1981 and pers. comm.), the protocols used in experiments on individual ionic effects have involved first removing salts by dialysis, in some instances at high pH and in the presence of EDTA, and then adding salts in the desired ratios. Even at physiological pH and without EDTA, this procedure causes at least some dissociation of the native Hc polymer to its monomeric subunits. Following complete dissociation and the readdition of critical ions the monomers reassemble again, but often to polymers smaller than the native molecule and with an "incorrect" subunit composition that alters O_2 affinity and its ion dependence (*e.g.* Jeffrey and Treacy, 1980). Thus it is not entirely clear that the ion sensitivities of the native arthropod hemocyanins are as diverse as implied by the literature.

The suggestion that divalent cations are responsible for the influence of total salinity (Truchot, 1975) seemed difficult to reconcile with the observations on *C. sapidus*. Either the divalent cation sensitivity of HcO_2 affinity is much greater in this strong regulator, or other factors are also involved. In addtion, an experiment performed by Mangum and Towle (1977) seemed difficult to reconcile with the important finding that the L-lactate produced during hypoxia raises HcO_2 affinity (Truchot, 1980), an effect that has now been demonstrated in *C. sapidus* as well as several other decapods (Johnson and Becker, 1981; Booth *et al.*, 1982; Graham *et al.*, 1982). Mangum and Towle (1977; Fig. 3) illustrated the responses of the HcO_2

transport system to the estuarine environment by comparing animals acclimatized to dilute and also moderately hypoxic waters with different animals acclimatized to more saline and also normoxic waters. Since the difference in HcO_2 affinity (3–4 mm Hg) could be reproduced *exactly* by altering the total salinity and pH of the blood of an individual animal, they attributed it to pH and salinity *per se*. According to Johnson and Becker (1981 and pers. comm.), however, even the small amounts of lactate produced during hypoxia should raise HcO_2 affinity by several fold more than observed.

We have investigated the basis of the salinity effect on HcO_2 affinity, and also other respiratory properties of the blood, in *Callinectes sapidus* Rathbun, using 1) procedures that do not alter the size or the subunit composition of the native polymer, 2) preparations stripped of dialyzable factors such as lactate, and 3) in an *in vivo* experiment, salinity alone as a stimulus of the change in the same individuals. We have identified the specific and the physiologically important inorganic ion effectors, and we have demonstrated an additional factor that dominates the response and thus explains several of the apparent contradictions in the literature.

MATERIALS AND METHODS

Collection and maintenance of animals

The effects of inorganic ions on HcO_2 binding were first examined using blood of crabs purchased during March, April and May from large commercial suppliers in the lower Chesapeake Bay. Animals were held for less than 24 h in recirculating water at 18‰ and 21–23°C, a period during which a temperature change has no effect (Mauro and Mangum, 1982). In most experiments, the blood of 27–36 individuals was pooled and the pool used for a related set of experiments; an exception in which paired observations were made on the same individuals is described in detail below. The animals were adult males in intermolt stage C ranging in carapace length from 6.0 to 7.3 cm.

Preparation of hemocyanin

Blood was extracted from the infrabranchial sinuses of each walking leg, allowed to clot in a tissue homogenizer, and then the clot was broken and separated from the serum by centrifugation. Ten ml aliquots of the pooled sera were dialyzed at 5°C against 1 l of the test solution (see below), buffered with 0.05 *M* Tris Maleate. The medium was changed after 24 h and the dialysis continued for an additional 24 h. The preparation was centrifuged again prior to the O_2 binding measurements.

Preparation of test solutions

Since our purposes were to investigate the effects of maximum physiological changes in inorganic ions and to distinguish clearly the effective from the ineffective ions in the blood, the entire physiological range found in nature was examined (Mangum and Amende, 1972; Lynch *et al.*, 1973; Colvocoresses *et al.*, 1974). We should emphasize that the physiological range is estimated from acute measurements (*i.e.*, made on freshly collected animals), and therefore they exaggerate the average physiological variation. This point is considered further in the Discussion.

The test solutions were prepared so that the activity of each ion in the single salt solutions and in the mixtures of two or three salts closely approximates the value in a graded series of physiological salines containing all of the major inorganic

TABLE I

Ionic composition of the complete physiological salines against which C. sapidus *blood was dialyzed*[1]

	Concentration (meq/l)							
	Solution 1		Solution 2		Solution 3		Solution 4	
Ions	Total	Free	Total	Free	Total	Free	Total	Free
Na^+	138	90	278	181	410	270	546	359
K^+	4	2.5	8	5.0	12	7.5	16	10.0
Ca^{+2}	15	2.6	25	4.8	35	7.0	45	9.2
Mg^{+2}	15	1.1	25	1.8	35	2.5	45	3.3
Cl^-	150	94	300	190	450	283	600	377
HO_3^-	2	1.2	2	1.2	2	1.2	2	1.2
SO_4	20	1.0	30	1.4	40	2.8	50	3.3
Total Salinity	342	194	664	386	984	574	1304	764

[1] The concentration of the free ions in the single and multiple salt solutions approximate the concentration of the free ions in the complete salines. Solutions were buffered with 0.05 *M* Tris Maleate.

ions found *in vivo.* The activity coefficients, given in detail by Mason (1982), were taken from Robinson and Stokes (1970) and from Pytkowicz *et al.* (1975). The composition of the series of physiological salines is given in Table I. The concentrations of free Na^+, K^+, Ca^{+2}, Mg^{+2} and Cl^- in the dialysate, or the product of total concentration and the activity coefficient, were ascertained by direct measurement with ion selective electrodes, using 1:99 dilutions of buffered, stirred samples. Further details of this procedure are described by Graham *et al.* (1982).

Oxygen binding measurements

Oxygen binding measurements were initiated within 30 min of completing the dialysis, using physiological concentrations of the protein. The sample was allowed to equilibrate for 10 min at atmospheric pressure with each of 4–7 humidified mixtures of N_2 (ultra high purity, scrubbed further in a 120 × 3 cm column of Ridox) and either O_2 (purity 99.5%) or air (scrubbed of CO_2 with KOH). The mixtures were prepared with a Wösthoff gas mixing pump. During the equilibration period, the vessels were immersed in a water bath (25°C), and the samples mixed. At each PO_2, changes in absorbance at 335 nm (1 mm light path) were noted with a Bausch and Lomb Spectronic 20 colorimeter.

Measurements of pH were made at 0 and 100% HcO_2. A Fisher Accumet 520 pH meter and a Radiometer electrode were used. The pH of the oxygenated and deoxygenated Hc preparations never differed by more than 0.02 pH units.

Two to four replicate measurements were performed on each preparation, and the data treated as a homogeneous sample. P_{50} and n_{50} values were determined from logarithmic regression lines fit to Hill plots of the pooled data in the range 15 to 85% HcO_2. The significance of differences between the values for n_{50} were estimated from the 95% confidence intervals around the slope and, for P_{50}, from 95% confidence belts around the regression lines.

Experimental design

The experiments testing the effects of different concentrations of individual ions and salt mixtures on HcO_2 binding at a common pH were designed so that the

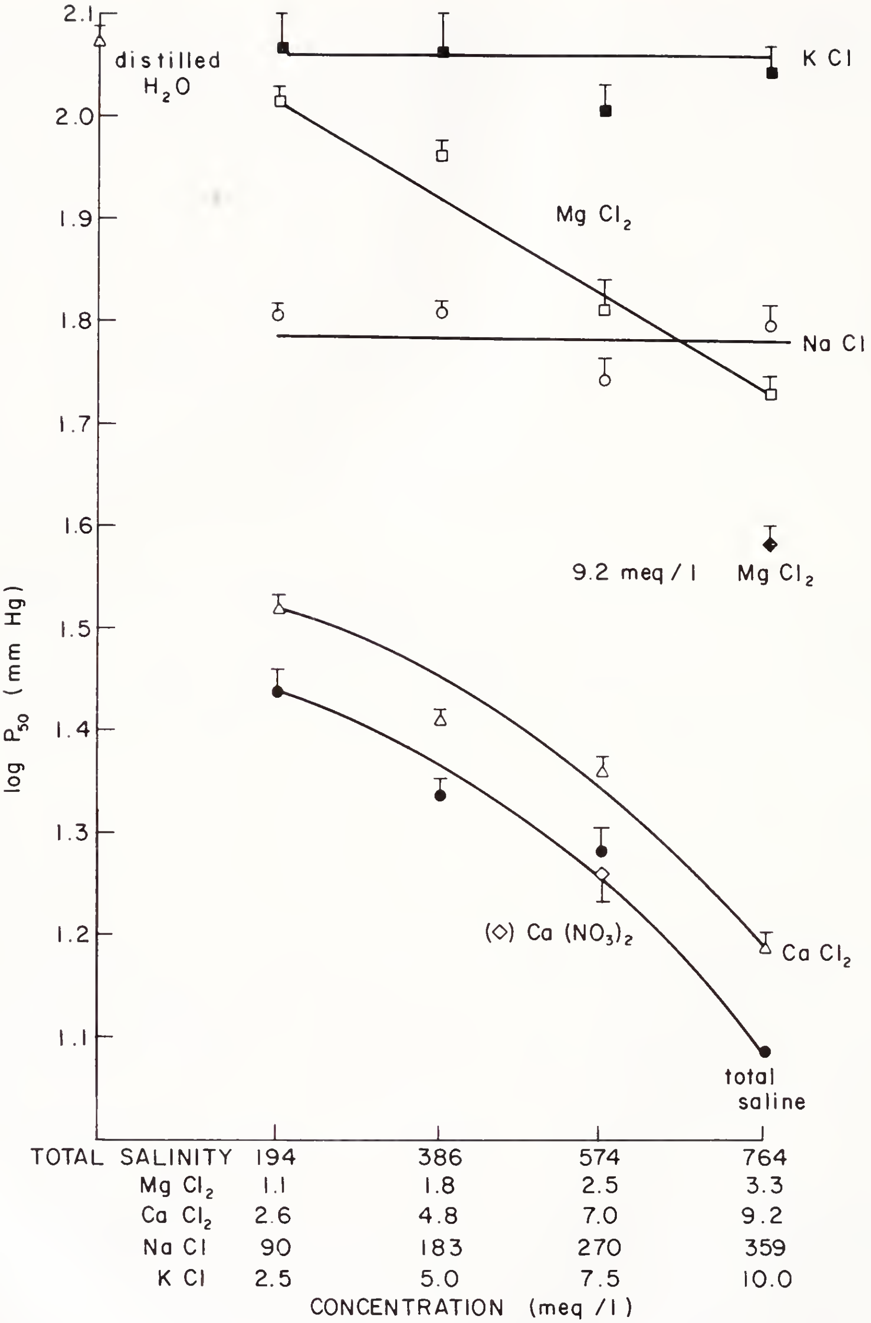

FIGURE 1. Effects of a complete physiological saline and of single inorganic salts on O_2 affinity (P_{50}) of *C. sapidus* Hc in 0.05 *M* Tris Maleate (pH 7.50 ± 0.22) at 25°C. Free ion concentration is given. (◇) 7.0 meq/l Ca $(NO_3)_2$. Bars show 95% confidence interval.

response of P_{50} and n_{50} to total salinity is the control, and the response to the test ions corresponding to a particular salinity is the experiment. For example, in Figure 1 the point at 194 meq/l salinity is the control for 1.1 meq/l $MgCl_2$, 2.6 meq/l

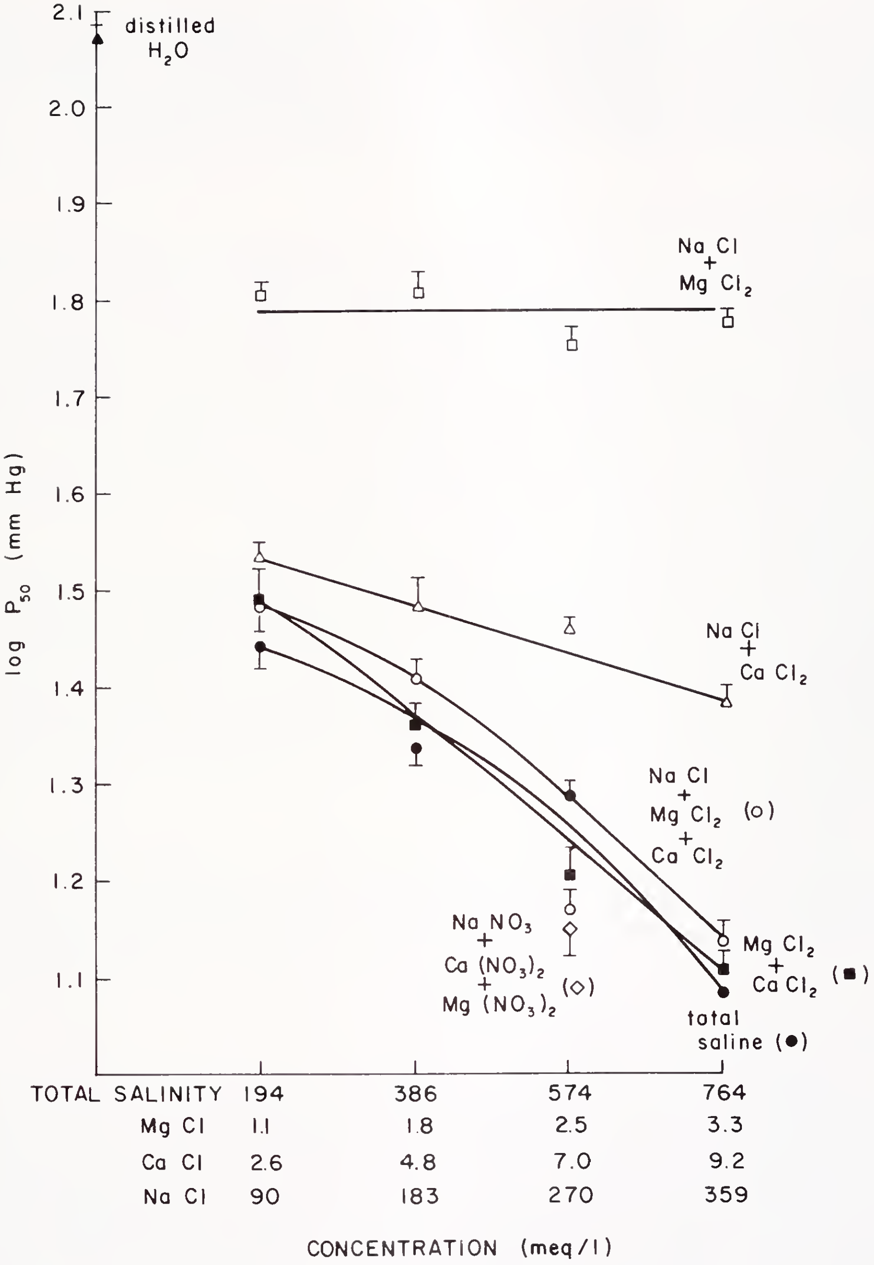

FIGURE 2. Effects of mixtures of inorganic salts on O_2 affinity (P_{50}) of *C. sapidus* Hc in 0.05 *M* Tris Maleate (pH 7.50 ± 0.02) at 25°C. Free ion concentration is given. Curve for complete physiological saline (●) is reproduced from Figure 1. (◇) 270 meq/l $NaNO_3$, 7.0 meq/l Ca $(NO_3)_2$, 215 meq/l Mg $(NO_3)_2$. Bars show 95% confidence interval.

$CaCl_2$, 90 meq/l NaCl and 2.5 meq/l KCl. A different pool of blood was used at each of the four sets of concentrations shown in Figures 1 and 2. Therefore, each point obtained at the different concentrations and thus connected by the curves represents a different pool of blood.

The experiments testing the effects of critical ions and salt mixtures on the Bohr shift were designed so that the response of the factor $\Delta \log P_{50}/\Delta pH$ to total salinity is the control and the response to single salts or a mixture of two salts is the experiment. The data obtained at each particular concentration and thus the points connected by the curves in Figures 3–6 represent the same pool of blood.

Acclimation to high and low salinity

Adult, intermolt males were purchased in June 1981 from a commercial supplier who had caught them in the upper York River estuary (0–3‰ salinity, measured with a Yellow Springs Instrument Co. conductivity meter) within the previous 6 h. Adult, intermolt males were also captured in pots by the authors in inlets of the Atlantic Ocean near Wachapreague, Virginia (34‰). While we cannot exclude the possibility of exchange between the two populations, the distance would require a travel period of several weeks. Each group was held for less than 12 h in aerated, natural water (21–23°C), the low salinity group at 5–8‰ (York River estuary water) and the high salinity group at 35‰ (Wachapreague Inlet water). Two ml of blood were taken from each crab; the crab was then transferred to the alternative salinity, held there for 8 days without feeding, and sampled again.

Half of the blood from each crab taken prior to and following the transfer was dialyzed against a complete physiological saline representing high salinity, and the other half was dialyzed against a saline representing low salinity. Oxygen binding measurements were made as described above and the Hc concentration estimated from the absorbance of [HcO_2–Hc] at 335 nm (Nickerson and Van Holde, 1971), under each of the four conditions. The data were analyzed as paired observations according to Student's t-test, using the 0.05 level as the criterion of significance.

Subunit composition

Polyacrylamide gel electrophoresis was performed according to the method of Davis (1964), using slabs of 7.5% acrylamide gel (1.5 mm thick), and 25–30 μg Hc, stripped by dialysis against 0.05 *M* Tris buffer containing 0.01 *M* EDTA (pH 8.9).

RESULTS

The effect of experimental design

As indicated above, the experiments on the effects of inorganic ions on HcO_2 binding at a common pH were designed so that each point connected by the curves in Figures 1 and 2 represents a different pool of blood, of necessity taken from animals collected during different seasons and, possibly, from different salinities in the range about 0–25‰. In view of the seasonal changes in HcO_2 affinity demonstrated in this species earlier (Mauro and Mangum, 1982) and the effects of acclimation salinity demonstrated below, appreciable scatter around the curves would be expected. In contrast, the experiments on the effects of inorganic ions on the Bohr shift were designed so that the points connected by the curves in Figures 3–6 were obtained from a single pool of blood. Considerably less scatter around the curves would be expected. These expectations are realized in the results.

Since acclimation does not appear to influence cooperativity in this species, the error around the values in Tables II and III should be unrelated to experimental design.

TABLE II

The effect of inorganic ions on cooperativity (n_{50}) of HcO_2 binding in C. sapidus *Hc*[1]

Solution	Free ion concentration (meq/l)	n_{50} (mean ± S.E.)
Distilled water	0	2.9 ± 0.2
Complete salines	194	3.0 ± 0.3
	386	3.2 ± 0.4
	574	3.4 ± 0.4
	764	2.9 ± 0.2
Single salts:		
NaCl	90	3.2 ± 0.2
	183	2.8 ± 0.2
	270	2.7 ± 0.3
	359	3.0 ± 0.3
KCl	2.5	2.6 ± 0.8
	5.0	1.7 ± 0.9
	7.5	2.5 ± 0.2
	10.0	2.6 ± 0.3
$CaCl_2$	2.6	2.5 ± 0.5
	4.8	3.6 ± 0.4
	7.0	3.1 ± 0.4
	9.2	2.8 ± 0.3
$MgCl_2$	1.1	2.9 ± 0.2
	1.8	3.3 ± 0.2
	2.5	3.2 ± 0.6
	3.3	3.8 ± 0.6
	9.2	3.4 ± 0.4
$Ca(NO_3)_2$	7.0	2.8 ± 0.4
Salt mixtures:		
$NaCl/MgCl_2$	90/1.1	3.3 ± 0.2
	183/1.8	3.8 ± 0.3
	270/2.5	3.1 ± 0.2
	359/3.3	3.6 ± 0.2
$NaCl/CaCl_2$	90/2.6	3.3 ± 0.2
	183/4.8	3.8 ± 0.4
	270/7.0	3.1 ± 0.3
	359/9.2	3.7 ± 0.2
$CaCl_2/MgCl_2$	2.6/1.1	3.3 ± 0.2
	4.8/1.8	3.7 ± 0.4
	7.0/2.5	3.1 ± 0.6
	9.2/3.3	2.8 ± 0.3
$NaCl/MgCl_2/CaCl_2$	90/1.1/2.6	3.6 ± 1.8
	183/1.8/4.8	3.6 ± 0.5
	270/2.5/7.0	3.1 ± 0.3
	359/3.3/9.2	2.9 ± 0.6
$NaNO_3/Mg(NO_3)_2/Ca(NO_3)_2$	270/2.5/7.0	2.8 ± 0.2

[1] 0.05 *M* Tris Maleate, 25°C, pH 7.48–7.52. *N* = 2–4.

The effect of total salinity

When the preparation is dialyzed against buffered distilled water, HcO_2 affinity becomes very low (Figs. 1 and 2), but cooperativity remains unchanged (Table II). When the preparation is dialyzed against a physiological saline containing all of the major ions found in the blood, HcO_2 affinity increases with an increase in total salt concentration (Figs. 1 and 2). Cooperativity, however, does not change (Table II).

The effect of single salts

At activities similar to those in the complete salines, no single inorganic salt present by itself clearly raises HcO_2 affinity in full to the level observed in the complete salines (Fig. 1). In the presence of small amounts of buffered KCl, the response does not differ significantly from that observed after dialysis against buffered distilled water. The presence of buffered NaCl (or $MgCl_2$) does increase HcO_2 affinity but, above 90 meq free Na^+/l, P_{50} appears to be independent of NaCl concentration. By far the most important single salt is $CaCl_2$, which very nearly restores HcO_2 affinity to the control levels. Indeed, the difference between the data for $CaCl_2$ and for the complete physiological saline, although significant, is very small. Ca^{+2} has a specific effect, an inference supported by the value at 9.2 meq/l $MgCl_2$; at this level, the free Mg^{+2} concentration approximates the highest free Ca^{+2} concentration used. In addition, Mg^{+2} has a much smaller effect than Ca^{+2} at similar activities (Fig. 1). At virtually equal activities, there appears to be a difference between the effects of $CaCl_2$ and $Ca(NO_3)_2$, but it is very small.

Increasing the concentrations of single salts at pH 7.5 has no significant effect on cooperativity (Table II).

The effects of salt mixtures

In the presence of two or three salts, the responses of P_{50} are not always simple sums of the single salt values (Figs. 1 and 2). In the presence of NaCl and $MgCl_2$, HcO_2 affinity behaves very much as it does in the presence of NaCl alone (Fig. 2). Mixing NaCl with $CaCl_2$ appears to mitigate the effect of $CaCl_2$. The addition of $MgCl_2$ to either $CaCl_2$ or the mixture of NaCl and $CaCl_2$ produces a result virtually indistinguishable from that of the complete saline. In these results the replacement of Cl_2 with NO_3^- in the mixture of Na^+, Mg^{+2} and Ca^{+2} has no detectable effect on P_{50} (Fig. 2). Regardless of the mixture, n_{50} does not change (Table II).

The effects of inorganic ions on the Bohr shift

When the preparation is dialyzed against distilled water, the Bohr shift is virtually eliminated ($\Delta \log P_{50}/\Delta pH = -0.16$ in the pH range 6.97–8.07; Fig. 3). However, in the most dilute physiological saline, the Bohr shift is restored in full (the Bohr factor, $\Delta \log P_{50}/\Delta pH = -0.99$ in the pH range 6.95–8.03) and it does not increase further in more concentrated physiological salines (Fig. 3). While the Bohr factor appears to increase with $MgCl_2$ concentration (Fig. 4), it remains the same in the presence of various levels of $CaCl_2$ alone, or in a mixture of the two (Figs. 5 and 6).

In contrast to the small difference observed at pH 7.5 (Fig. 1), the more extensive observations in Figures 3 and 5 indicate that the presence of $CaCl_2$ alone restores HcO_2 affinity to the control level, at least in the range 7.0–9.2 meq free Ca^{+2}/l, and that the presence of Mg^{+2} is not necessary.

Although n_{50} generally appears to reach a maximum at about pH 7.8 (Table III),

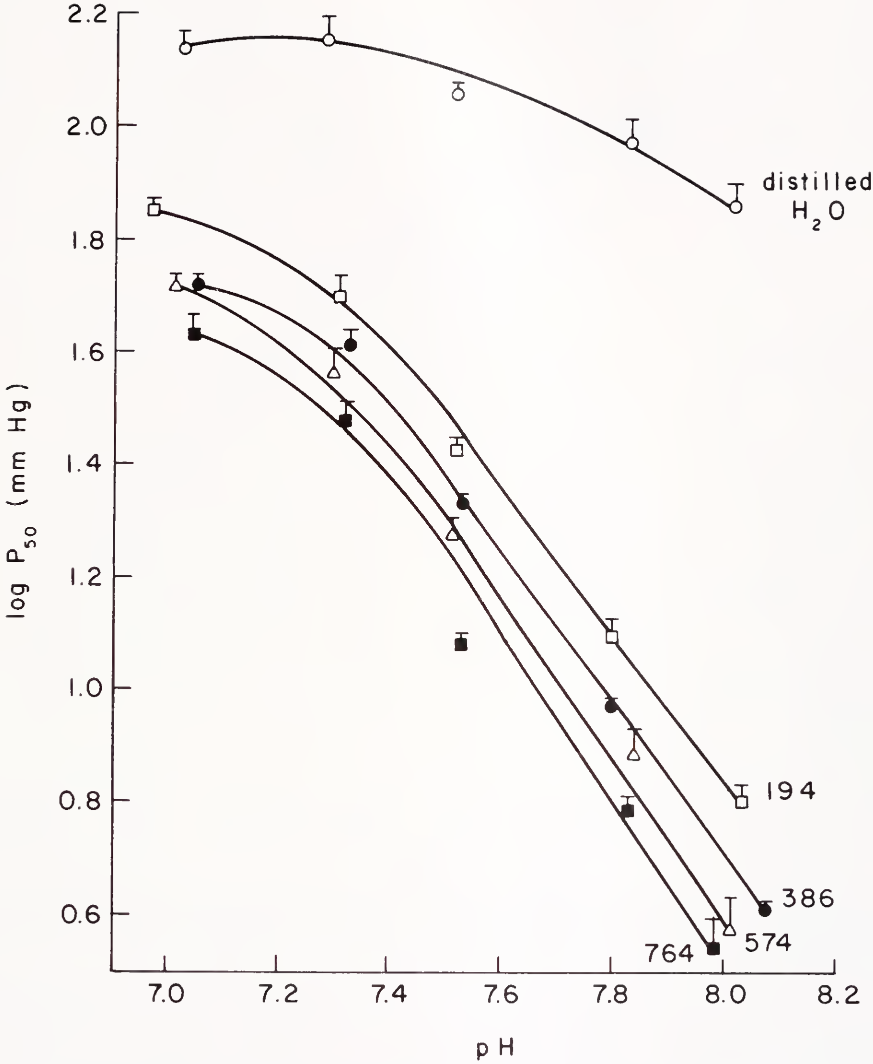

FIGURE 3. Effect of total salinity on the Bohr shift of *C. sapidus* Hc in 0.05 *M* Tris Maleate at 25°C. Curves are designated by free ion concentration. Bars show 95% confidence interval.

the trend is not significant, in part because of the small number of observations in each data set. Only in the presence of pure $MgCl_2$, where n_{50} continues to rise throughout the range examined, does pH significantly influence cooperativity (Table III).

The effect of salinity acclimation

Animals collected at high and low salinities have Hcs with significantly different O_2 affinities (Fig. 7). The high salinity population has a higher HcO_2 affinity than the low salinity population. The difference disappears completely 8 days after transfer of the low salinity crabs to high salinity, and it disappears in a large part 8 days after transfer of the high salinity crabs to low salinity. In the latter case, the trend

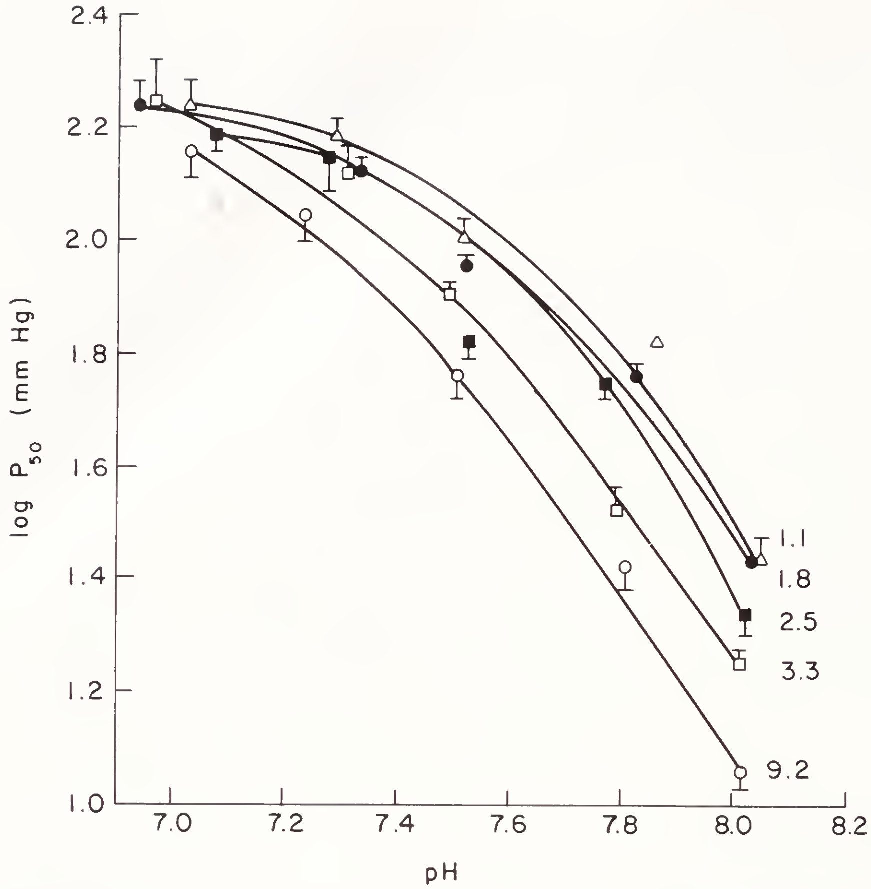

FIGURE 4. Effect of $MgCl_2$ on the Bohr shift of *C. sapidus* Hc in 0.05 *M* Tris Maleate at 25°C. Curves are designated by free ion concentration. Bars show 95% confidence interval.

may reflect individual differences since only one of the six points is significantly different. No change in cooperativity was detected (Table IV; Fig. 7).

The data in Table V suggest a change in Hc concentration, namely an increase at low salinity and a decrease at high salinity. However, the differences are significant only in the group transferred from high to low salinity.

Preliminary observations on the subunit composition of the hemocyanins suggest that, depending on resolution, as few as five or as many as seven electrophoretically separable polypeptides can be detected in both populations. Of 25 pools used in the investigation of inorganic ion effects, all obtained from Chesapeake Bay crabs, 23 are alike; they are also like a pooled preparation of the samples taken from the low salinity crabs used in the acclimation experiment, but they differ from a pool of the samples taken from the high salinity crabs used in the acclimation experiment.

Specifically, bands three and five are far more concentrated in the low than in the high salinity pool (Fig. 8). After making the oxygen binding measurements, material was available for electrophoresis only from the group transferred from low

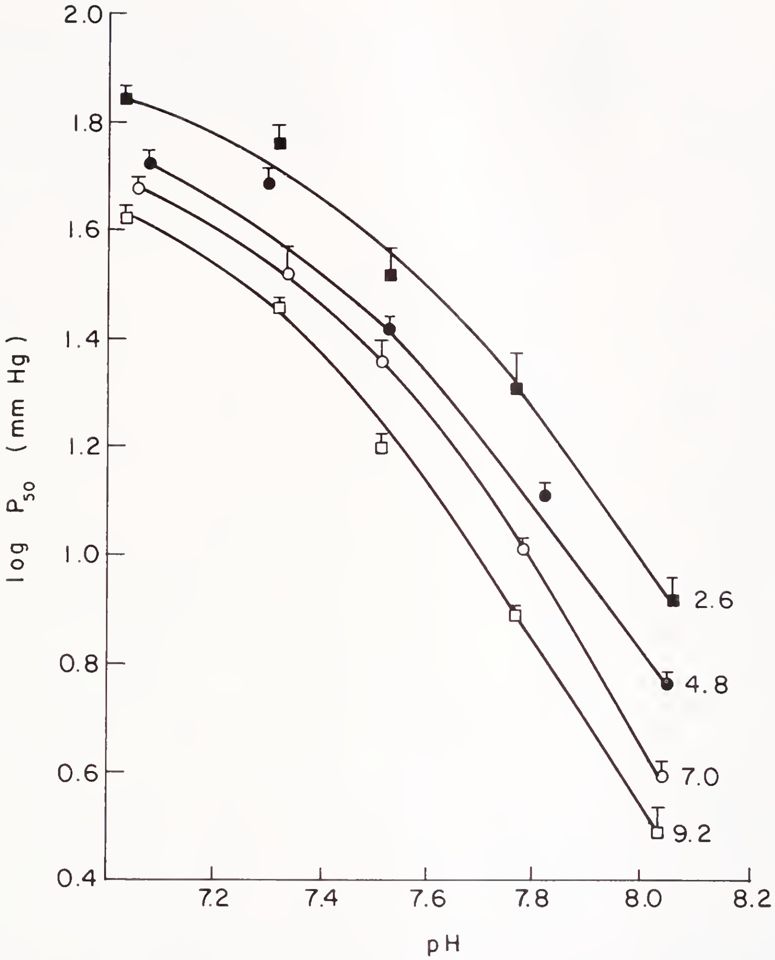

FIGURE 5. Effect of $CaCl_2$ on the Bohr shift of *C. sapidus* Hc in 0.05 *M* Tris Maleate at 25°C. Curves are designated by free ion concentrations. Bars show 95% confidence interval.

to high salinity. Perhaps more interesting, this pool was no longer exactly like the one obtained prior to the transfer; instead, bands three and five had become less concentrated, the pattern appearing intermediate between the two populations (Fig. 8). This observation was made in replicate.

DISCUSSION

The labile oxygenation properties and their inorganic effectors

The O_2 affinity of *C. sapidus* Hc rises measurably when the total salinity increases within the physiological range. The levels of K^+ in the blood are apparently too small to have a detectable effect, and the effects of Na^+ are moderate, reaching their maximum either at or below 90 meq free Na^+/l. Increases in Ca^{+2} and Mg^{+2}, on the other hand, continue to raise HcO_2 affinity throughout the physiological range.

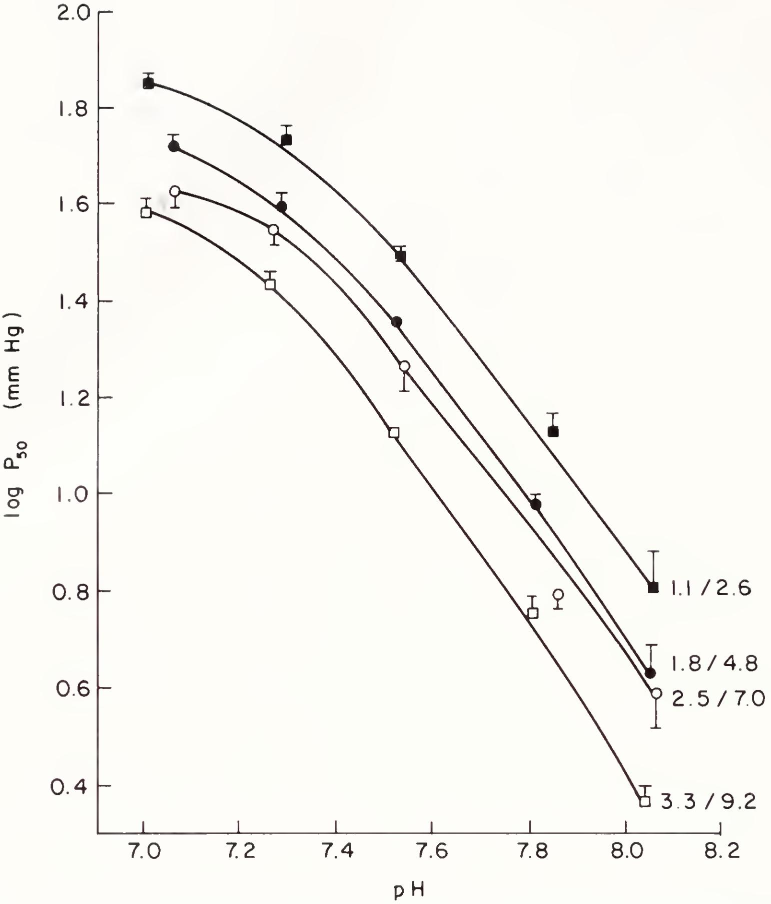

FIGURE 6. Effect of a mixture of $MgCl_2$ and $CaCl_2$ on the Bohr shift of *C. sapidus* Hc in 0.05 *M* Tris Maleate at 25°C. Curves are designated by free ion concentration, with $MgCl_2$ given first. Bars show 95% confidence interval.

Of the two, Ca^{+2} is clearly the more important, by a factor approaching six. A factor describing Ca^{+2} sensitivity (Δ log P_{50}/Δ log $[Ca^{+2}]$) is almost three times greater in *C. sapidus* (−0.82; Fig. 5) than in *C. maenas* (−0.28; Truchot, 1975). Indeed, our data (Figures 3 and 5) suggest that, as long as free Ca^{+2} exceeds 7 meq/l, no other inorganic ion is required to restore HcO_2 affinity to the level found in a complete physiological saline.

While the effect of Ca^{+2} is very large and highly specific, a specific Cl^- effect is either absent or so small that it cannot be clearly discerned. The magnitude of the change in Hc molecular weight following the removal of Ca^{+2} at physiological pH (7.8) (Herskovits *et al.*, 1981) suggests that little, if any, of the Ca^{+2} effect results from dissociation of the native dodecamer. The absence of a Ca^{+2} effect on cooperativity also supports this conclusion.

Using chelating agents to remove divalent cations, other investigators (Chantler *et al.*, 1973) have reported an effect of Ca^{+2} on the cooperativity of portunid crab

TABLE III

The effect of inorganic ions and pH on cooperativity (n_{50}) of HcO_2 binding in C. sapidus[1]

Sample	Free ion concentration (meq/l)	pH (±0.02)	n_{50} (mean ± S.E.)
Distilled water	0	7.01	2.2 ± 0.3
		7.27	2.3 ± 1.0
		7.51	3.0 ± 0.2
		7.82	2.6 ± 0.3
		8.00	3.3 ± 0.8
Complete salines	194	6.97	2.6 ± 0.3
		7.30	2.8 ± 0.5
		7.49	3.0 ± 0.3
		7.79	3.9 ± 0.9
		8.03	3.1 ± 0.3
	386	7.04	2.6 ± 0.6
		7.30	2.8 ± 0.8
		7.49	3.2 ± 0.4
		7.79	3.6 ± 0.5
		8.06	2.3 ± 0.8
	574	7.00	2.7 ± 0.3
		7.29	2.8 ± 0.7
		7.50	3.4 ± 0.2
		7.83	3.5 ± 0.6
		8.01	2.8 ± 0.8
	764	7.03	3.0 ± 0.7
		7.32	3.1 ± 0.5
		7.52	3.0 ± 0.2
		7.82	3.8 ± 0.6
		7.98	2.7 ± 0.8
$CaCl_2$	2.6	7.02	2.9 ± 0.2
		7.31	2.7 ± 0.4
		7.52	2.5 ± 0.6
		7.76	2.8 ± 0.5
		8.05	2.7 ± 0.9
	4.8	7.06	2.6 ± 0.9
		7.29	3.2 ± 0.6
		7.49	3.6 ± 0.4
		7.81	3.4 ± 0.3
		8.04	3.8 ± 0.6
$CaCl_2$	7.0	7.05	2.8 ± 0.3
		7.33	2.2 ± 0.8
		7.49	3.1 ± 0.4
		7.78	3.1 ± 0.4
		8.04	2.6 ± 0.6
	9.2	7.03	2.5 ± 0.2
		7.31	3.1 ± 0.3
		7.50	2.8 ± 0.3
		7.76	3.2 ± 0.2
		8.03	2.9 ± 0.4

TABLE III (*Continued*)

Sample	Free ion concentration (meq/l)	pH (± 0.02)	n_{50} (mean ± S.E.)
$MgCl_2$	1.1	7.02	1.8 ± 0.3
		7.28	2.2 ± 0.2
		7.50	2.9 ± 0.2
		7.85	3.9 ± 0.3
		8.05	3.4 ± 0.8
	1.8	6.95	1.9 ± 0.4
		7.32	2.5 ± 0.3
		7.48	3.3 ± 0.2
		7.82	3.9 ± 0.3
		8.03	3.8 ± 0.4
	2.5	7.06	2.2 ± 0.2
		7.27	2.4 ± 0.6
		7.52	3.2 ± 0.4
		7.77	4.0 ± 0.4
		8.02	4.1 ± 0.5
	3.3	6.96	2.0 ± 0.6
		7.31	2.7 ± 0.6
		7.50	3.5 ± 0.4
		7.79	4.0 ± 0.3
		8.01	4.5 ± 0.6
	9.2	7.02	2.3 ± 0.5
		7.24	3.2 ± 0.4
		7.50	3.8 ± 0.7
		7.80	4.2 ± 0.4
		8.01	4.8 ± 0.5
$MgCl_2/CaCl_2$	1.1/2.6	7.00	3.0 ± 0.2
		7.28	2.8 ± 0.3
		7.49	3.3 ± 0.2
		7.84	3.5 ± 1.1
		8.05	2.6 ± 0.7
$MgCl_2/CaCl_2$	1.8/4.8	7.05	3.2 ± 0.2
		7.28	2.8 ± 0.6
		7.50	3.7 ± 0.4
		7.80	3.3 ± 0.4
		8.04	2.4 ± 0.8
	2.5/7.0	7.05	2.5 ± 0.3
		7.25	2.6 ± 0.3
		7.49	3.1 ± 0.6
		7.85	3.9 ± 0.8
		8.06	2.5 ± 1.0
	3.3/9.2	6.99	3.2 ± 0.4
		7.26	3.1 ± 0.5
		7.51	2.8 ± 0.3
		7.81	3.0 ± 0.9
		8.04	2.5 ± 0.9

[1] 0.05 *M* Tris Maleate, 25°C. $N = 2$–4.

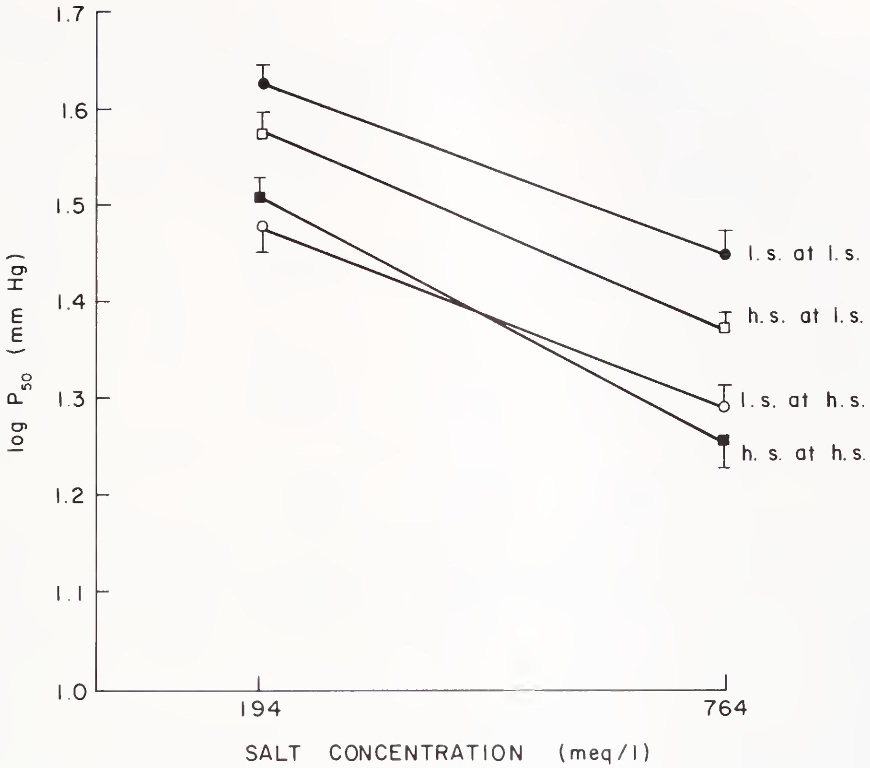

FIGURE 7. The effect of acclimation salinity on O_2 affinity of *C. sapidus* Hc (0.05 *M* Tris Maleate) at 25°C and pH 7.53 ± 0.02. Low salinity (0–3‰) population (l.s. at l.s.), low salinity population after 8 days at 35‰ (l.s. at h.s.), high salinity (34‰) population (h.s. at h.s.), and high salinity population after 8 days at 5–8‰ (h.s. at l.s.). Blood was dialyzed against 194 and 764 meq/l complete physiological saline solutions. Paired observations were made ($N = 6$ for the low salinity population, $N = 7$ for the high salinity population). Bars show 95% confidence interval.

HcO_2 binding with the physiological pH range (6.9 to 8.1). At pH 10, the removal of Mg^{+2} causes complete dissociation to monomers (Hamlin and Fish, 1977), which would eliminate cooperativity. However, the present findings clearly indicate that changes of orders of magnitude in excess of the physiological range are necessary to influence cooperativity in *C. sapidus*. The present results also indicate that very little Ca^{+2} is required to maintain the Bohr shift at the physiological level, even less than the amount needed to restore HcO_2 affinity. Thus, HcO_2 affinity is the only oxygenation property that responds to changes in the inorganic ions in the blood.

The role of the unidentified effector

Although highly variable, the acute measurements of blood calcium made on freshly collected crabs by Colvocoresses *et al.* (1974) suggest that the average value at 0‰ would be about 27 meq/l, and the average value at 35‰ would be about 34 meq/l. At 27 meq total Ca/l, l (= *ca.* 5.3 m*M* free Ca^{+2}/l) P_{50} would be about 27 mm Hg (pH 7.5; Fig. 1); at 34 meq total Ca^{+2}/l (= 6.8 m*M* free Ca^{+2}/l), P_{50} would be 23 mm Hg. The average changes in Ca^{+2} would alter HcO_2 by 4 mm Hg; hence

TABLE IV

Cooperativity (n_{50}) of HcO_2 binding in native and acclimated C. sapidus *populations after dialysis against 194 meq/l or 764 meq/l complete physiological salines*[1]

Sample	Free ion concentration (meq/l)	n_{50} (mean ± S.E.)
Low salinity population (0–3‰)	194	3.0 ± 0.2
(N = 6)	764	3.5 ± 0.3
Low salinity population after 8 days at 35‰	194	3.4 ± 0.5
(N =6)	764	3.2 ± 0.9
High salinity population (34‰)	194	3.3 ± 0.5
(N = 7)	764	3.4 ± 0.3
High salinity population after 8 days at 5–8‰	194	3.8 ± 0.7
(N = 7)	764	3.3 ± 0.4

[1] 0.05 *M* Tris Maleate (pH 7.51–7.55), 25°C.

the direct effect of total salinity on the blood of an individual observed by Mangum and Towle (1977). However, the physiological response of the HcO_2 transport system is far more complicated than previously supposed. At pH 7.5 and low salt concentration the actual difference in HcO_2 affinity between populations acclimated to normoxic water and to salinities only slightly less different than 0 and 35‰ is about 43–32 = 11 mm Hg (Fig. 7); at high salt concentration, the difference is about the same (29–18.5 = 10.5 mm Hg). Thus the effect of low salinity acclimation on HcO_2 affinity is considerably larger than that attributed by Mangum and Towle (1977) to the salt effect alone. The increment is due to changes in a non-dialyzable factor which are induced, in large part or in full, within 8 days.

Because the data were obtained from dialyzed preparations, the most probable explanation of the acclimation might seem to be a change in the protein *per se*, resulting in Hcs with intrinsically different O_2 affinities in the high and low acclimation states. Two lines of evidence might be invoked to support this suggestion: the increase in total hemocyanin concentration and the increase in concentration of two of the 5–7 subunits at low salinity. In our view, however, neither set of evidence is particularly cogent.

Using unpaired observations on different individuals held at high and low salinity, a number of investigators have examined the relationship in portunid crabs between environmental salinity and either total protein concentration (Horn and Kerr, 1963; Lynch and Webb, 1973; Péqueux *et al.*, 1979) or Hc concentration

TABLE V

The effect of acclimation salinity on Hc concentration (mean ± S.D.) in C. sapidus[1]

Sample	Hc concentration (g/100 ml)
Low salinity population (0–3‰) (N = 6)	5.9 ± 1.8
Change in low salinity population after 8 days at 35‰ (N = 6)	−3.2 ± 2.3
High salinity population (34‰) (N = 7)	6.1 ± 1.3
Change in high salinity population after 8 days at 5–8‰ (N = 7)	+2.2 ± 0.8

[1] Paired observations on the same individuals before and after transfer to the alternative salinity.

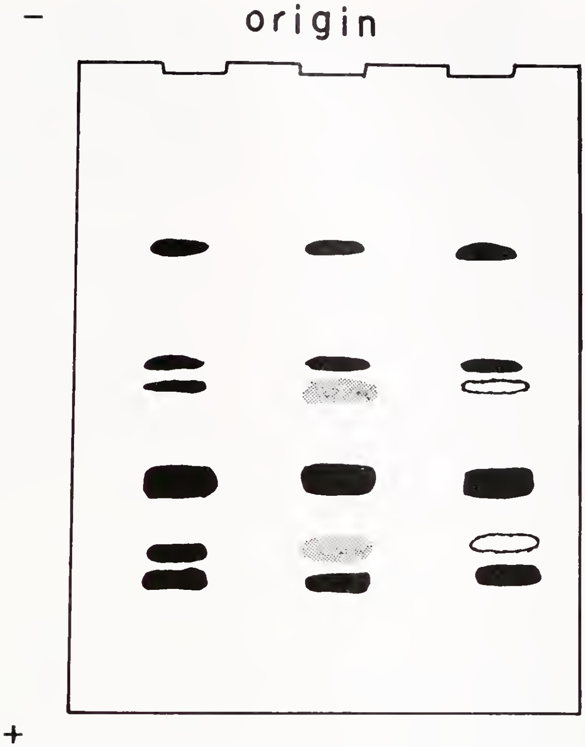

FIGURE 8. The effect of acclimation salinity on the concentration of electrophoretically separable polypeptides of *C. sapidus* Hc. Left to right: Low salinity population, low salinity population after 8 days at 35‰, and high salinity population.

(Boone and Schoffeniels, 1979). Boone and Schoffeniels (1979) and Péqueux *et al.* (1979) both reported extremely large, though not significant, changes that could be invoked to support an hypothesis of *de novo* Hc synthesis at low salinity. Horn and Kerr (1963) mention no clear relationship between salinity and either protein or Cu concentration, and the more numerous observations on serum protein in freshly collected animals also show no clear trend (Lynch and Webb, 1973). The enormous variation among different individuals may preclude a very firm conclusion from the previous investigations.

Our own data, based on paired observations following transfers in both directions and thus eliminating individual variation as well as differences in nutritional state, indicate that a net increase in synthesis, if it occurs at all, is smaller than reported previously (Péqueux *et al.*, 1979). Since blood volume remains essentially constant over a wide salinity range (Robinson, 1982), a net change in Hc synthesis would not be masked by concomitant changes in extracellular space. On the other hand, it is likely that an increase in synthesis would be masked by a concomitant increase in degradation to keep the Hc concentration from exceeding a level that can be efficiently handled by the cardiovascular system (Snyder and Mangum, 1982). Thus the available information on Hc concentration argues neither for nor against the replacement of one Hc molecule by another as the mechanism of low salinity acclimation.

Although the role of subunit heterogeneity in assembling the hemocyanin polymers is presently becoming clear (*e.g.* Markl and Kempter, 1981), its physiological significance, if any, has yet to be investigated. The shift in subunit ratios following acclimation of *C. sapidus* to a new salinity is certainly an interesting observation, the meaning of which will be pursued by determining the frequencies of the two phenotypes in the high and low salinity populations, and by correlating the shift with HcO_2 affinity on an individual basis. However, we should point out that the shift, which was incomplete, was observed in the group in which the acclimation of HcO_2 affinity was complete.

Regardless of the mechanism, the significance of the salinity acclimation is very clear. In the laboratory, brief (8 h) exposure to hypoxic water results in large increases (>5 m*M*) in blood lactate (Mangum, unpublished data). If unopposed by concomitant changes in other effectors of HcO_2 binding, these levels could raise HcO_2 affinity by a factor large enough to impair O_2 delivery to the tissues. In fact, the conjunction of pH, lactate, Ca^{+2} and salinity acclimation effects may prove to account for the smaller increase in HcO_2 affinity observed in freshly caught estuarine animals by Mangum and Towle (1977) and attributed to inorganic ions alone. The actual balance achieved by the various effectors of HcO_2 affinity awaits further investigation of animals acclimated to both dilute and moderately hypoxic water.

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α-LACTOSE BINDING HEMAGGLUTININS FROM THE ASCIDIAN *PHALLUSIA MAMILLATA* (CUV.)

NICOLÒ PARRINELLO AND CALOGERO CANICATTÌ

Zoological Institute, Palermo University, Via Archirafi, 18, 90123 Palermo, Italy

Abstract

The carbohydrate binding specificities of *Phallusia mamillata* anti-rabbit and anti-human (A, B, O) hemagglutinins were determined by hemagglutinin inhibition tests. Both these molecules bind α-lactose, while only anti-HE hemagglutinins bind lactulose. In spite of this difference, absorption experiments did not show two distinct agglutinins for rabbit and human erythrocytes. Since both sugars are oligosaccharides which present a D-galacto-configuration, isolation by affinity chromatography with Sepharose was performed. The D-galactose-eluted fraction, investigated by PAGE and SDS-PAGE, appears to be the more anodal serum glycoprotein which consists of 2 subunits: the larger has a molecular weight of approximately 61–65,000 Daltons and the second one was 4000 Daltons smaller than the first. Gel filtration on Sephadex G 200 suggests that hemagglutinins represent a population of molecules in which the larger molecules (<200,000 Daltons) represent the greatest fraction.

Introduction

The presence of hemagglutinins has been demonstrated in worms, molluscs, arthropods, echinoderms and protochordates (Tyler and Metz, 1945; Tyler, 1946; Wright and Cooper, 1975; Parrinello and Patricolo, 1975; Amirante, 1976; Parrinello *et al.*, 1976). These molecules seem to be synthesized by hemocytes (Amirante, 1976) in the hemolymph; they can interact with foreign substances and show opsonic properties (Prowse and Tait, 1969; McKay and Jenkin, 1970; Cooper, 1976), forming a simple recognition system which enables invertebrates to discriminate between "self" and "non self". They are usually high molecular weight substances which appear to be protein made up of subunits forming large molecules which in no way resemble the immunoglobulins of the vertebrates (Tyler and Metz, 1945; Tyler and Scheer, 1945; Cohen, 1968; Acton *et al.*, 1969; Hall and Rowlands, 1974a; Pauley, 1974; Parrinello and Patricolo, 1975; Parrinello *et al.*, 1976). Their binding sites have specificity for carbohydrate moities, and several distinct subpopulations of active molecules can bind different sugars (Johnson, 1964; Sharon and Lis, 1972; Gold *et al.*, 1974; Hall and Rowlands, 1974b; Ryoyama, 1974; Anderson and Good, 1975; Hammarstrom *et al.*, 1977; Parrinello and Canicattì, 1982).

The sera of both lower and higher vertebrates also contain naturally occurring proteins which exhibit binding sites for carbohydrates (cf. Litman, 1976). These molecules can be immunoglubulins (Sela *et al.*, 1975; Karajalainen and Mäkelä, 1976) or non-immunoglobulin lectin-like proteins (Harisdangkul *et al.*, 1972; Litman, 1976). Since tunicates represent a phylogenetically critical taxon (Berril, 1955),

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Abbreviations: PBS = phosphate buffered saline; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulfate; RE = rabbit erythrocytes; HE = human erythrocytes; HA-RE = erythrocyte-bound hemagglutinin.

studies of their sugar-specific agglutinins could contribute to an evaluation of the structural and functional evolution of these proteins.

The blood of ascidians contains hemagglutinins which react with vertebrate erythrocytes and bind the carbohydrate components on the cell surface (Anderson and Good, 1975; Wright and Cooper, 1975; Marchalonis and Warr, 1978; Parrinello and Canicattì, 1982). The agglutinin specificity has usually been determined by competitive binding studies involving the capability of monosaccharides or their derivatives to inhibit the agglutination of erythrocytes by agglutinin (Sharon and Lis, 1972). In a previous paper we showed that in the serum of *Ascidia malaca* two distinct agglutinins with similar sugar specificity ranges were responsible for rabbit and human erythrocyte agglutination (Parrinello and Canicattì, 1982). The specificity range includes saccharides containing a D-galacto-configuration (D-melibiose, D-raffinose, D-galactose, α-lactose, lactulose, L-arabinose) and allowed the isolation of the agglutinating serum fraction by biospecific affinity chromatography of the serum with Sepharose. *Phallusia mamillata* Cuv. possesses protein substances which agglutinate a variety of erythrocytes (Parrinello and Patricolo, 1975). The present report is concerned with the specificity of these hemagglutinins as determined by competitive binding studies and with the electrophoretic and immunological characterization of the isolated serum fraction.

MATERIALS AND METHODS

Phallusia mamillata were collected in the Gulf of Palermo. The blood, obtained by cardiac puncture, was centrifugated at 400 × *g*. The pooled serum, dialyzed with phosphate buffered saline at pH 7.4 (PBS), was divided into 2 ml aliquots and stored at −20°C.

Serial doubling dilutions of serum were prepared in U-bottom wells of microtiter plates (Cooke Engineering Co., Alexandria, Virginia) following the standard method (Kabat and Mayer, 1961). Hemagglutinating activity was determined with a suspension of washed erythrocytes (8×10^7 cells/ml). The agglutination trays were incubated for one h at 37°C and the assay read after 12 h at 4°C, the end titer being taken as the reciprocal of the last dilution giving a clear agglutination following gentle shaking.

The erythrocyte suspension (4×10^8 cells/ml, in PBS) was incubated with trypsin or bromelain (Sigma, 0.1 mg/ml) for 30 min at 37°C. The treated erythrocytes were washed 5 times with PBS.

Absorption studies were carried out as follows: packed erythrocytes (2 ml) and equal volumes of serum preparations were mixed and incubated at room temperature for 1 h, and at 4°C for 12 h, with occasional shaking. The absorbed serum was obtained by centrifugation of the mixture.

Hemagglutination inhibition was tested by the same method used for *A. malaca* agglutinins (Parrinello and Canicattì, 1982). Dialyzed serum (0.25 ml) was added to 0.05 ml of a 2-fold serial dilution of carbohydrates using microtiter plates. After a 30 min incubation at 37°C, 0.025 ml of erythrocyte suspension (8×10^7 cells/ml) were added. The hemagglutination test was carried out under the above mentioned conditions and the last sugar dilution capable of inhibiting hemagglutination was evaluated.

Protein content was determined by the Folin-Ciocalteau method (Lowry *et al.*, 1951). Bovine serum albumin was the reference standard. Each value was expressed as average of three determinations ± SE.

Gel filtration experiments were performed using a 1 × 60 cm Sephadex G-200 column equilibrated with PBS. Serum samples were concentrated by ultrafiltration in a Diaflo equipped with a UM 2 membrane (Amicon Corp., Lexington, MA) and then dialyzed overnight against the starting buffer. One ml of three-fold concentrated serum was loaded onto the column at 20°C. At a flow rate of 8 ml per h, 0.5 ml fractions were collected, read for UV (280 nm) absorbancy, and tested for hemagglutinating activity. Dextran blue 2000 (Pharmacia), human IgG, bovine serum albumin and cytochrome C (Sigma) were used to calibrate the column (Andrews, 1965).

Affinity chromatography column (1.5 × 15 cm) were filled with acid-treated Sepharose CL-6B (Pharmacia) prepared according to Ersson *et al.* (1973). The gel suspended in 0.2 M HCl was stirred at 50°C for 3 hours and after several washings, equilibrated with PBS. After a 20 ml load of serum the columns were washed with 10–12 bed volumes of NaCl (1 *M*) and then eluted with 0.2 *M* sugar in NaCl (1 *M*). The sugar-eluted fractions, dialyzed against NaCl (1 *M*) and PBS to remove the sugar, and the unspecific eluted fractions were assayed for hemagglutination activity with rabbit (RE) and human A, B, O erythrocytes (HE).

Electrophoresis in 7.5% polyacrylamide gels (PAGE) was performed as described by Davis (1964). The sample (50 μl) was added to an equal volume of 5 m*M* Tris-glycine, pH 8.3, containing 40% sucrose and the mixture was deposited on the spacer gel. A constant current of 1 mA/tube was supplied until the buffer line passed through the spacer gel into the small-pore gel. The current was then increased to 3 mA/tube and the electrophoretic run was carried out for 60 min. Proteins were stained for 1 h with Coomassie Blue. Following electrophoresis, color reactions of the gels were performed to investigate the glycoprotein or lipoprotein nature of the isolated active fraction. Periodic acid-Schiff's reagent (Felgenhauer, 1970) and Sudan Black staining (Prat *et al.*, 1969) were used as described by Maurer (1971).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Shapiro *et al.*, 1967; Weber and Osborne, 1969) was carried out as described by Gavery *et al.* (1977). Gels containing 5.6% acrylamide and 0.2% *N*,N-methylenbisacrylamide were used. The samples were dialyzed against 0.02 *M* Tris-acetate buffer containing 2% β-mercaptoethanol and 2% SDS and then heated for ten min at 100°C. Phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme (Bio-Rad) were the molecular weight protein standards for SDS gel electrophoresis. The electrophoretic run was carried out for 4 h at 4 mA/tube.

Antiserum to rabbit erythrocyte-bound hemagglutinins was produced preparing the antigen according to Amirante (1976). *P. mamillata* serum was absorbed with RE as previously described. The agglutinated erythrocyte mass (HA-RE), obtained by centrifugation of the mixture, was washed several times with PBS to remove serum components. The rabbit which had supplied the erythrocytes for absorption was injected as follows: 5 ml of HA-RE emulsified with an equal volume of Difco Freund's complete adjuvant divided in small doses was injected intramuscularly into both hind legs; a second intramuscular injection identical to the first was given on day 30; subcutaneous injection of 5 ml of HA-RE in PBS was given in two sites on day 60. The animal was bled from an ear vein on day 40 and by cardiac puncture on day 70. The sera were not pooled but kept in separate 2 ml lots and stored at −20°C.

Microimmunoelectrophoresis (Scheidegger, 1955; Ouchterlony and Nilsson, 1973) was carried out using 1.5% agarose (Bio-Rad) and Trisbarbital sodium barbital buffer, pH 8.8. A current of 4 mA/strip was applied for 50 min. Antiserum diffusion was allowed to occur in a moist chamber at room temperature generally for 24 to 72 h. Dried agarose films were stained with Coomassie Blue.

TABLE I

Hemagglutinating activity of Phallusia mamillata *serum against untreated and protease-treated erythrocytes*

	Hemagglutinating titer[2] (reciprocal)	
Treatment[1]	Rabbit Erythrocytes	Human Erythrocytes (A, B, O)
—	32–128	4–16
Trypsin	254–2048	32–128
Bromelain	254–2048	254–2048

[1] Erythrocytes incubated with enzyme (0.1 mg/ml) for 30 min at 37°C.

[2] Hemagglutination titer ranges obtained from repeated tests and expressed as reciprocal of the last serum dilution giving agglutination.

RESULTS

Hemagglutinating activity of the serum against untreated and treated erythrocytes. Absorption experiments

The *P. mamillata* serum showed a high agglutinating activity against both rabbit and human A, B, O erythrocytes. As shown in Table I, the hemagglutination titers of the anti-RE (32–128) were higher than those of the anti-HE (4–16). The protease treatments enhanced the susceptibility of erythrocytes to agglutination: bromelain was more effective than trypsin in increasing the susceptibility of RE and HE to agglutination by *P. mamillata* agglutinins.

To verify the occurrence of specific hemagglutinins, the absorption experiments were performed. Sera showed no residual activity when absorbed with either RE or HE (A, B, O groups).

Sugar specificity of the anti-RE and anti-HE agglutinins

Carbohydrate binding specificity of *P. mamillata* anti-RE agglutinin was examined using various monosaccharides, oligosaccharides and modified sugars to inhibit hemagglutination of the untreated erythrocytes. The experiments showed that the specificity of the agglutinins is limited to α-lactose which inhibits the agglutination reaction up to final concentration of 71.5 μ moles/ml, SE ± 14 (Table II). No significant inhibition was observed with the monosaccharides D-mannose, D-glucose, D-fructose, L-sorbose, D-ribose and D-xylose; the oligosaccharides D-melezitose, maltose, D-cellobiose, D-trehalose, D-melibiose, and D-raffinose and lactulose; the modified sugars D-digitoxose, 2-deoxyglucose, 2-deoxyribose, salicin, sialic acid, N-acetyl-D-mannosamine, lactobionic acid and the two p-nitrophenyl-(α or β)-D-galactosides, when tested at final concentrations (0.4 M) higher than necessary to obtain complete inhibition with α-lactose. The polyalcohols D-mannitol, D-sorbitol, myo-inositol and dulcitol were also inactive.

Each of the above listed sugars were also tested for inhibition of agglutinating capability of the serum with human A, B, O erythrocytes. It was found that α-lactose inhibits the agglutination reaction at a lower concentration than that required to inhibit anti-RE agglutination. Lactulose interacts weakly with anti-HE agglutinins as a high concentration was required to inhibit agglutination (Table II).

Divalent cations are not necessary for carbohydrate-agglutinin binding as shown by the results of the inhibition experiments in which reaction mixtures containing

TABLE II

α-Lactose and lactulose inhibition of the agglutination of rabbit and human erythrocytes with Phallusia mamillata *serum preparations.*

Sugar	Minimum sugar concentration (μ mole/ml) for inhibition[1]			
		Human erythrocyte		
	Rabbit erythrocytes	A	B	O
α-lactose	71.5 ± 14.4	50.5 ± 17.8	28.7 ± 10.7	25.0 ± 10.5
lactulose	—	167.4 ± 33.0	144.0 ± 56.8	147.7 ± 54.3

[1] Average values ± SE obtained by testing erythrocytes from 7 specimens.
— = sugar ineffective at maximum concentration (0.4 *M*) used for inhibition.

EDTA (0.01–0.1 *M* final concentration, pH corrected to 7.4) were tested with HE and RE.

Sugar-agglutinin binding is a reversible process. Serum preparations incubated with α-lactose at the maximum concentration used in this study show agglutinating capability after exhaustive dialysis with saline solution.

Gel column chromatography of the serum

The protein content determinations of serum samples concentrated by ultrafiltration have shown that the hemagglutinating titer of the *P. mamillata* serum is related to the protein concentration: 167 ± 1.5 μg protein per ml produces a hemagglutinating titer of 16–32; ten-fold concentrated serum (525 ± 1.8 μg protein/ml) gave a titer of 128–256.

To determine the relative size of the molecules responsible for the hemagglutinating activity, gel filtration of the concentrated serum (525 ± μg protein/ml) on a calibrated Sephadex G-200 column (1 × 60 cm) was performed. Figure 1a shows a typical protein elution profile in which the agglutinating activity against RE and HE appeared in the single major protein fraction eluted with the blue dextran. Figure 1b shows the column chromatography separation of serum previously frozen for 8–12 months. Three separate peaks were obtained; hemagglutinating activity was mainly associated with the major protein fraction which was eluted at 13.5 ml with the blue dextran, however hemagglutinins were also found in the fractions eluted at volumes ranging between 15 and 25 ml which include the elution volumes of human IgG (160,000 Daltons) and ovalbumin (43,000 Daltons). This suggests that the hemagglutinins in a long-time frozen and concentrated serum were associated with molecules heterogeneous in size.

Affinity chromatography of the agglutinins

The demonstration of the reactivity of *P. mamillata* anti-RE and anti-HE agglutinins with oligosaccharides containing D-galactose (α-lactose and lactulose) and the availability of D-galactose support (Sepharose, Pharmacia) led us to explore the use of this medium for purification of the agglutinins. In a typical separation experiment, anti-RE and anti-HE agglutinins were isolated from 20 ml of whole serum (protein content 358 ± 1.1 μg per ml) on a column of cross-linked acid-treated Sepharose 6B (Fig. 2). After this treatment a greater number of end groups became available for reaction with agglutinins (Ersson *et al.*, 1973), which are adsorbed by

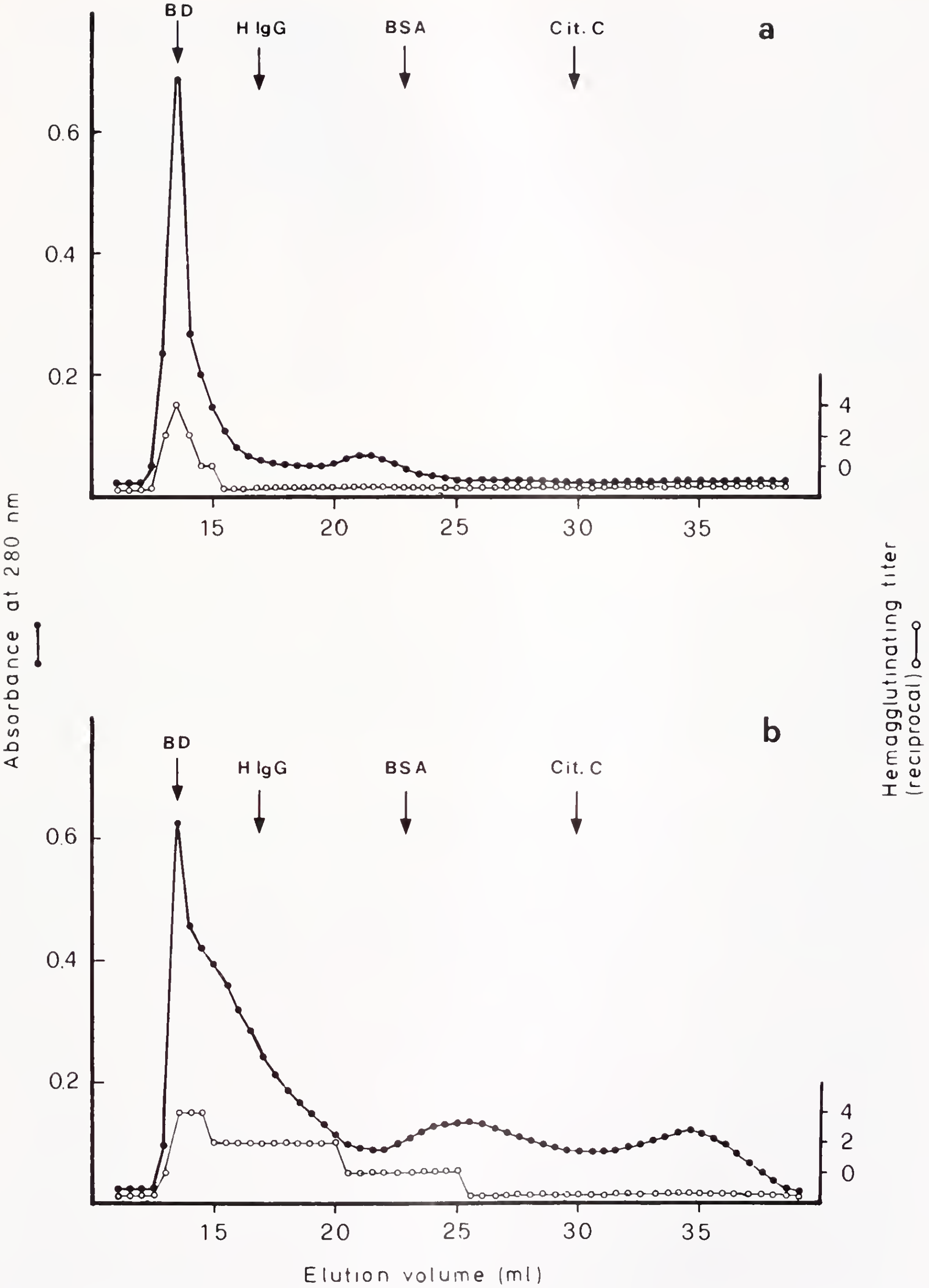

FIGURE 1. Sephadex G-200 chromatography of *Phallusia mamillata* concentrated serum (protein content ~1 mg/ml). The column (1 × 60 cm) was equilibrated and eluted with PBS. The flow rate was 8 ml/h, 0.5 ml fractions were collected. The elution positions of Blue Dextran 2000 (BD), human IgG (HIgG), bovine serum albumin (BSA), and citochrome C (Cit. C) are indicated. The hemagglutinating titers, expressed as reciprocal of the serum dilution, were obtained using RE and HE (A, B, O). In "b" the elution profile of serum frozen for 8–12 months is shown.

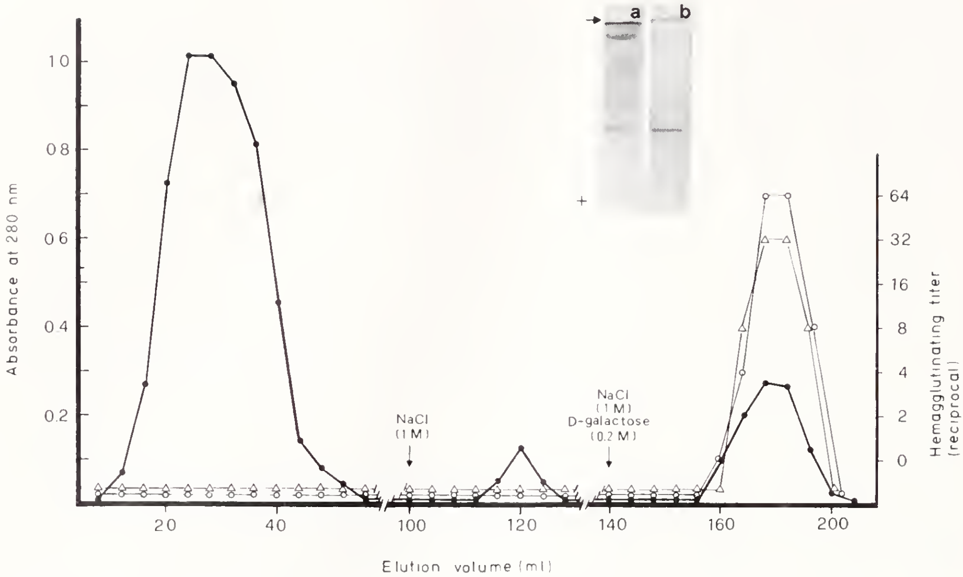

FIGURE 2. Affinity chromatography of *Phallusia mamillata* hemagglutinins on acid-treated Sepharose CL-6B. PBS-dialyzed serum (20 ml) was applied to a column (1.5 × 15 cm) at 25°C. The column was washed with 1 *M* NaCl and, at the point indicated, elution was started with 1 *M* NaCl plus 0.2 *M* D-galactose. The flow rate was 20 ml/h. The eluted fractions were dialyzed against NaCl (1 *M*) and PBS to remove the sugar. All the fractions were assayed for hemagglutinating activity with RE and HE, hemagglutinating titers were expressed as reciprocal of the serum dilution. Inset: developed portions of the polyacrylamide gels showing electrophoretic patterns; a) whole serum, b) pooled D-galactose eluted fraction showing maximum hemagglutinating titer. Arrow indicates the origin.

the gel bed. The first peak observed in the elution profile contains all the inactive proteins. The column was then washed with 1 *M* NaCl to elute the non-agglutinating fractions from Sepharose. Finally, when the eluate had reached a constant low absorbance at 280 nm, anti-RE and anti-HE agglutinins were displaced from the Sepharose bed by a solution of D-galactose (0.2 *M*) in 1 *M* NaCl.

These experiments clearly demonstrate that 0.2 *M* D-glucose in 1 *M* NaCl solution, a non-inhibitor of the agglutinin-polysaccharide interaction, did not elute agglutinins specifically bound to Sepharose, whereas α-lactose, like D-galactose, (0.2 *M* solution containing 1 *M* NaCl) readily displaced them from the gel bed. NaCl solution (1 *M*) or sugar in PBS (0.2 *M* solution) were unable to elute the agglutinins.

Electrophoretic and immunological characterization of the isolated agglutinins

The whole serum and the D-galactose-eluted protein were investigated by polyacrylamide gel electrophoresis. In Figure 2 the patterns of the *P. mamillata* serum (Fig. 2a) and the isolated fraction (Fig. 2b) are compared; Coomassie blue staining revealed that the agglutinating activity was principally connected with the large, more anodal protein component of the serum. Color reaction of the gels with periodic acid-Schiff's reagent after electrophoresis showed the glycoprotein nature of this serum component.

To study the D-galactose-eluted fraction by immunological methods, antiserum against the agglutinin bound to RE was produced. The immunoelectrophoresis results presented in Figure 3 show that the anti-serum reacts with two distinct com-

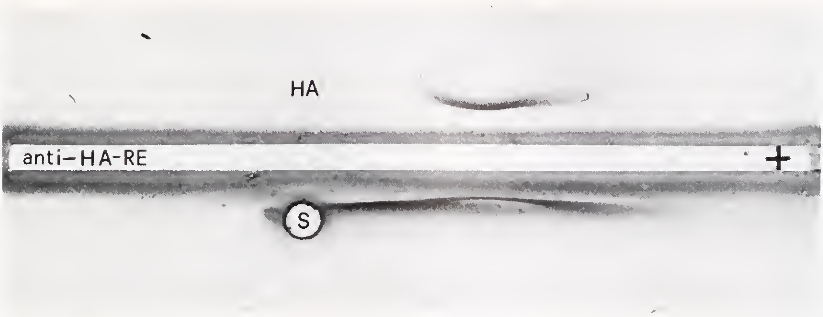

FIGURE 3. Immunoelectrophoresis in which antiserum, produced against rabbit erythrocyte-bound agglutinins (anti-HA-RE), reacted with D-galactose-binding hemagglutinin isolated by Sepharose affinity chromatography (HA) and normal *Phallusia mamillata* serum (S). The fractions used were those resulting in the maximum hemagglutinating titer.

ponents of the whole serum, whereas only the more anodal immunoprecipitates can be observed as a result of interaction between the antiserum and the D-galactose-eluted fraction. The morphology of the latter arc suggests that it represents several immunologically distinguishable components.

Molecular weight determination of the reduced agglutinins

In electrophoresis on SDS-polyacrylamide gel, the electrophoretic mobility of each protein chain is inversely proportional to the logarithm of its molecular weight (Shapiro *et al.*, 1967; Weber and Osborne, 1969). When D-galactose-eluted agglutinin preparations were reduced and denaturated by SDS, polyacrylamide gel electrophoresis showed that these proteins were dissociated into two subunits with similar molecular weights (Fig. 4a) determined from standard curves of the type shown in Figure 4b. The values were obtained from six independent experiments. The heavy chain ranged from 61,000–65,000 Daltons and the other subunit, in each determination, was always about 4000 Daltons lighter (mean values: 64,000 and 60,000 ± 1630 SD). Very low concentrations of low molecular weight protein chains ($21–23 \times 10^3$ and $35–40 \times 10^3$ Daltons) can occasionally be found, but they could be a result of the denaturating experimental conditions.

DISCUSSION

We have shown that naturally occurring hemagglutinins from *Phallusia mamillata* react with the sugar moities of human and rabbit erythrocyte membrane receptors. The inhibition data suggest that the anti-RE agglutinin presents a binding site which reversibly interacts with the α-lactose. The whole of this sugar molecule seems to be involved in the binding, in fact neither D-galactose nor D-glucose by themselves, nor other oligosaccharides containing these monosaccharides, are capable of inhibition. A D-galacto-configuration is also involved in the binding of the agglutinin with the HE: both α-lactose and lactulose (4-(β-D-galactosyl)-D-fructose) inhibit this agglutination. In spite of this specific binding of lactulose and of the different inhibition power of α-lactose, two different agglutinins for RE and HE could not be demonstrated by absorption experiments. These apparently contradictory results could be explained by the hypothesis that the variation in the inhibition powers of these sugars is caused by some slight difference in the binding sites

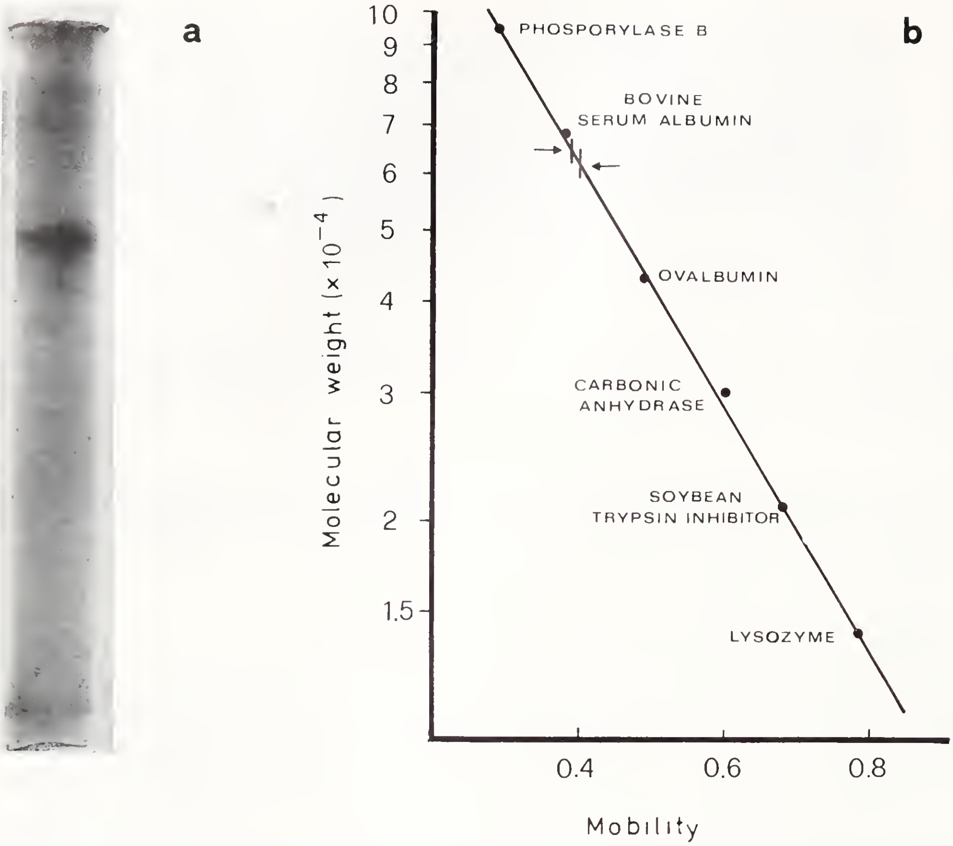

FIGURE 4. (a) SDS-polyacrylamide gel electrophoresis of *Phallusia mamillata* D-galactose-binding hemagglutinins isolated by Sepharose affinity chromatography. The eluted fractions used were those resulting in the maximum hemagglutinating titer. (b) Plot of molecular weight ($\times 10^{-4}$) against electrophoretic mobilities of different protein chains on SDS-polyacrylamide gel electrophoresis. The mobility was calculated according to Weber and Osborne (1969). The vertical bars indicate the estimated standard deviations of *Phallusia mamillata* hemagglutinin subunits (arrows) molecular weights.

of the hemagglutinins. D-galactose, while not involving divalent cations, seems principally responsible for the receptor-agglutinin interaction: agglutinin can bind to the end D-galactose groups of the acid-treated Sepharose and D-galactose solution can elute it; D-glucose was unable to elute the agglutinin even when in highly concentrated NaCl solution. A galactosyl group was apparently connected with recognition very early in phylogeny. D-galactose reactivity characterizes plant and invertebrate agglutinins from several sources (Johnson, 1964; Ersson *et al.*, 1973; Gold *et al.*, 1974; Ryoyama, 1974; Parrinello and Canicattì, 1982) and it could be a common character of a primordial self-nonself recognition mechanism. The particular spatial configuration of the galacto-pyranosyl structure makes this a prominent group which binds agglutinin even if it is contained in masked erythrocyte surface receptors, as suggested by the increased agglutinability of the protease-treated HE and RE.

Regarding the binding site, affinities exist between *P. mamillata* and *Ascidia malaca* hemagglutinins. Analysis of anti-RE and anti-HE *A. malaca* agglutinins (Parrinello and Canicattì, 1982) has suggested that the size of the combining site corresponds to a disaccharide with a specificity for saccharides containing a D-galacto-configuration. The differences involve the specificity range of *A. malaca* agglutinins which includes D-galactose, L-arabinose, D-melibiose, D-raffinose, α-lactose and lactulose.

A most convenient method which fulfills the requirements of isolation of agglutinins in highly purified form is affinity chromatography, which is based on the ability of agglutinins to bind carbohydrates specifically and reversibly. We have already reported the specific binding of *A. malaca* agglutinins to Sepharose gel and their elution by specific sugar (Parrinello and Canicattì, 1982). The *P. mamillata* anti-RE and anti-HE agglutinins were isolated by this method too, but a sugar solution of a high ionic strength was required to break the sugar-protein interactions. In this respect these agglutinins differ from those of *A. malaca* which were eluted by a D-galactose solution in PBS.

The isolated agglutinins seem to correspond primarily to the most anodal protein fraction of the serum when analyzed by polyacrylamide gel electrophoresis. The Schiff's reagent demonstrated the glycoprotein nature of this fraction. In immunoelectrophoresis it appears as a single electrophoretic band when tested with an antiserum versus erythrocyte-bound agglutinins, however the morphology of the immuno-precipitate suggest that it can be formed of several antigens. The ability of this antiserum to precipitate two different components from the whole serum could depend on the antigen preparation used for immunization or it could be due to cross reaction.

Molecular sieve column chromatography of the serum on Sephadex G-200 beads indicates that hemagglutinins can be large molecules with molecular weights greater than 200,000 Daltons. Most of the agglutinating activity appeared in the major protein fraction eluted with blue dextran. As shown by gel filtration experiments, freezing may alter the hemagglutinin enough to produce smaller agglutinating molecules. The elution profile of the frozen serum showed a smaller peak of activity a few tubes behind the blue dextran suggesting hemagglutinin size smaller than 160,000 Daltons; activity could also be found in the fractions containing proteins with molecular sizes about that of ovalbumin (43,000 Daltons). These findings suggest that the agglutinating molecules can be composed by subunits which associate to form macromolecular complexes.

Several papers indicate that invertebrate agglutinating sugar-specific proteins are different from agglutinating antibodies that vertebrates possess. Recently Marchalonis and Warr (1978) suggested that the subunits of the naturally occurring DNP-binding protein of the ascidian *Pyura stolonifera* resembles heavy chains (μ) of mammalian IgM when studied by PAGE analysis, in which their molecular weights appear to be $65–70 \times 10^3$ Daltons. We found that the subunits of the isolated sugar-specific agglutinins of *P. mamillata* are similar in size to those of *Pyura* and show medium molecular weight of 60,000 and 64,000. In this respect the ascidian Sepharose-isolated hemagglutinins could be a starting point for the study of the evolution of these molecules. Discussion of such a process will be possible when data on amino acid composition, carbohydrate content, and structural molecular organization can be evaluated.

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THE FEEDING ECOLOGY OF *HYDRA* AND POSSIBLE IMPLICATIONS IN THE STRUCTURING OF POND ZOOPLANKTON COMMUNITIES

STEVEN S. SCHWARTZ, BRENDA J. HANN,* AND PAUL D. N. HEBERT

Department of Biology, University of Windsor, Windsor, Ontario, Canada, N9B 3P4

ABSTRACT

Although most invertebrate predators are size-selective, two species of *Hydra*, *H. oligactis* and *H. pseudoligactis*, are not. A marked preference for *Daphnia pulex* over *Simocephalus vetulus*, similarly sized prey items, is observed in feeding trials. *S. vetulus* is virtually ignored and swims among the tentacles of *Hydra*, whereas *D. pulex* is rapidly attacked and captured. However, normal feeding responses are induced in the presence of homogenates of both *Daphnia* and *Simocephalus*. This suggests that the lack of response to live *Simocephalus* as prey items may be due to defense mechanisms evolved during the course of long-term coexistence in the shallow, weedy littoral zone of lakes and ponds. Such mechanisms could involve reduced activation of nematocysts, immunity to the toxin, or lack of penetration of nematocysts through the carapace of *Simocephalus*. The impact on the structure of the zooplankton community of this differential susceptibility to predation by *Hydra* is discussed.

INTRODUCTION

In the past 20 years it has become apparent that both vertebrate (Brooks and Dodson, 1965) and invertebrate (Dodson, 1974) predators are important in structuring zooplankton communities. In most instances only the larvae of the midge *Chaoborus* and copepods are considered as dominant invertebrate predators on pond zooplankton. Recently, however, other invertebrates, such as notonectids (O'Brien and Vinyard, 1978), dytiscids (Arts *et al.*, 1981), odonates (Johnson, 1973; Johnson and Crowley, 1980), and flatworms (Maly *et al.*, 1980; Schwartz and Hebert, 1982), have been recognized as having possible roles in shaping zooplankton communities. All of these invertebrate predators have been deemed 'size-selective' (Zaret, 1980) in that each demonstrates a marked preference for prey of a particular, usually small, size. By preferring small prey the predators constitute a selective pressure favoring species or individuals larger than the preferred feeding range (Zaret, 1980).

The cladoceran genera *Simocephalus* and *Daphnia* are commonly represented in pond habitats throughout the temperate zone. Although species of the two genera often co-exist, *Daphnia* tend to be most abundant in ponds lacking vegetation, while *Simocephalus* predominates in macrophyte-filled ponds. It has been argued that *Daphnia* avoid these latter habitats because of the presence of toxins released by the plants (Hasler and Jones, 1949; Pennak, 1973) or due to interference with swimming behavior from the vegetation (Porter, 1977), but the role of predation has not been critically assessed. The abundance of *Simocephalus* might simply reflect the presence of substrates for attachment or alternatively the resistance of this species to predators common in macrophyte-filled habitats.

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* Present address: Department of Zoology, University of Manitoba, Winnipeg, Manitoba R3T 2N2.

In an effort to understand the factors governing the distribution of these two genera, we set up a number of aquaria containing *Daphnia pulex* and *Simocephalus vetulus.* In agreement with the results of earlier laboratory studies (Frank, 1952), we found that *Daphnia* rapidly displaced *Simocephalus.* There is little doubt that *Simocephalus* is competitively inferior to *Daphnia* even in small containers in which the surface area:volume ratio is large. However, when *Hydra* were added to the aquaria the *Daphnia* population declined rapidly and *Simocephalus* became the numerical dominant. As adult *Daphnia* and *Simocephalus* are of similar size, it was clear that the *Hydra* were selecting prey not on the basis of size but on some other criteria. This initial observation led to the present research, the goals of which were to: 1) determine the selectivity of two *Hydra* species on cladoceran species of different behavior and size and 2) establish the basis of selection.

Stimulation for feeding and the feeding mechanics are both well known for *Hydra* (reviewed by Lenhoff, 1968). Predation is cued by an initial chemical stimulus which sensitizes the nematocysts. When the trigger, or cnidocil, is subsequently given mechanical stimulus the nematocyst is fired and the prey paralyzed and trapped (Lentz, 1966). For the species of *Hydra* studied here, two types of nematocysts were most frequently observed: piercing stenoteles and entangling desmonemes. By means of synchronous tentacular flexing and contraction (referred to as a concert), the prey is brought toward the mouth and slowly engulfed. The entire process takes less than two min for small prey such as *Artemia* nauplii (Rushforth and Hofman, 1972). Though this process is well understood there is sparse literature concerning the implications of *Hydra* feeding on natural prey assemblages and prey community structure (Cuker and Mozley, 1981; Schroeder and Callaghan, 1982).

MATERIALS AND METHODS

Two species of *Hydra* were used in the feeding trials: *H. oligactis* and *H. pseudoligactis. Hydra oligactis* was collected at Fish Lake, Lagrange County, Indiana, from an extensive bed of *Elodea* in water less than a meter deep. *Hydra pseudoligactis* was collected at Rondeau Provincial Park, Kent County, Ontario, in a shallow (less than 1 m), well-shaded forest pool. Species identifications were made using Hyman's key (1959) with the lengthwise coiling of the filament in the holotrichous isorhiza and lack of nipples on the testes clearly distinguishing *H. oligactis* from *H. pseudoligactis.* Populations of these species were established in synthetic pond water (Hebert and Crease, 1980) and fed a mixture of *Daphnia* and *Ceriodaphnia* at two day intervals. M solution, the frequently used medium for *Hydra,* was not used as it was found to be lethal to the cladocerans. The prey species used in the trials were all common cladocerans at Rondeau Park and included *Daphnia pulex, Daphnia laevis, Ceriodaphnia reticulata, Scapholeberis kingi,* and *Simocephalus vetulus.* The two larger species, *D. pulex* and *S. vetulus,* were divided into adult and juvenile size classes to provide two additional prey groups. All cladocerans were also cultured in synthetic pond water.

Feeding trials were conducted on laboratory reared *Hydra* by placing one *H. oligactis* (8–10 mm when fully extended) or two *H. pseudoligactis* (5–8 mm total length) in a 120 ml plastic cup with 100 ml synthetic pond water, a depth of 4 cm. Two individuals of *H. pseudoligactis* were used due to their smaller size. The *Hydra* were starved at least 24 h prior to the addition of 20 prey items to each cup. Trials were conducted with 8 replicates and two controls. At the end of 12 h at 20°C in constant light the remaining live prey were counted in each cup. In addition, selectivity was tested by providing 10 juveniles of both *D. pulex* and *S. vetulus* in similarly conducted trials.

TABLE I

ANOVA for feeding trials with Hydra.

	df	ms	F
Hydra oligactis			
Among prey	6	33.78	11.37**
Within prey	42	2.97	
Total	48		
Hydra pseudoligactis			
Among prey	6	246.57	23.51**
Within prey	42	10.49	
Total	48		

** $p < .0001$.

Behavioral responses were observed in 3-depression slides. Single *Hydra* were presented with several individuals of a prey species and the response noted over a 5 min interval. Different *Hydra* were used for each prey item. To observe the feeding response in the absence of behavioral differences in prey species, trials were conducted in which homogenates of prey species (produced by crushing a single individual of a prey species in a drop of synthetic pond water) were introduced into the region of the *Hydra* tentacles. Response to the homogenate was also observed during a 5 min interval. As a certain background rate of concerting may occur, two 5 min observation periods preceded every behavioral response trial. In the event that the background rate of concerting exceeded 1/min, that particular *Hydra* was discarded.

RESULTS

Feeding ecology

The results of feeding trials with two species of *Hydra*, fed 8 different species or combinations of prey species, are presented in Tables I and II. No prey items died in the control containers during the course of their feeding trials. The rank order of mean numbers of prey surviving to the end of the trial was the same for both *Hydra* species, indicating similar prey preferences, and showed no concordance with ranking of the prey items by length. Thus, these species of *Hydra* are not size-selective predators. No distinction was made between prey which had been actively consumed and those killed coincidentally as the result to the prey population is the same.

The prey species could be divided into two or more distinct groups based on their relative vulnerability to predation by *Hydra* (Table II, Duncan's multiple range test, $p < .05$). *Daphnia pulex* adults and juveniles and *Ceriodaphnia* proved to be very vulnerable to attack, whereas *Simocephalus vetulus*, adults and juveniles, and *Scapholeberis* were relatively immune. The response to *Daphnia laevis* differed for the two *Hydra* species: *H. pseudoligactis* preyed upon *D. laevis* with an intensity intermediate between the two groups described above, while *H. oligactis* attacked *D. laevis* as intensely as *Ceriodaphnia* and *D. pulex*. Both *Hydra* species attacked *D. pulex* juveniles at a rate approximately twice that for juvenile *S. vetulus* when presented with a choice of the two prey species ($t = 8.98$ and 5.625 for *H. oligactis* and *H. pseudoligactis*, respectively, $p < .0001$).

TABLE II

Mean length of prey times, mean number of prey items remaining alive at the end of feeding trials for two species of Hydra *and their grouping by Duncan's multiple range test (p < .05).*

Prey	Age Class	Mean (±s.e.) Length (mm)	Mean (±s.e.) Number of prey alive			
			H. pseudoligactis	Group	*H. oligactis*	Group
Simocephalus vetulus	A	1.70 ± .02	19.6 ± .2	a	20	a
Simocephalus vetulus	I	.66 ± .01	18.2 ± .5	a b	20	a
Scapholeberis kingi	A	0.80*	18.1 ± .5	a b	19.3 ± .2	a
Daphnia laevis	A	1.58 ± .03	15.4 ± .6	b	17.2 ± .5	b
Ceriodaphnia reticulata	A	.69 ± .01	10.2 ± 1.0	c	17.2 ± .9	b
Daphnia pulex	A	1.88 ± .03	7.7 ± .7	d	15.6 ± .8	b
Daphnia pulex	I	1.20 ± .02	5.7 ± 1.2	d	15.0 ± .5	c
Daphnia pulex and	I		4.2 ± .5		6.1 ± .6	
Simocephalus vetulus			9.7 ± .1		9.6 ± .2	

* Length data from Brooks, 1959.
Means with the same letter are not significantly different. A = adult, I = immature.

Behavioral responses

Responses of the *Hydra* to the various prey items fell into three distinct categories. The immediate response of a *Hydra* to the introduction of *D. pulex* individuals was the gentle waving of its tentacles in the approximate direction of the prey. As soon as the prey contacted one or more of the tentacles, its swimming movements slowed and the animal appeared to be quickly paralyzed. The *Hydra* then either directed the prey toward the mouth by tentacular movements, or occasionally the prey dropped out of reach and was ignored. When the homogenate of a crushed daphniid was introduced near the *Hydra* tentacles, there was an initial burst of concerts, followed by a steady decline in rate of concerting over the 5 min period of observation, the average rate being 5–6 concerts/min.

In contrast was the response of *Hydra* when *Scapholeberis* was presented as prey, where rapid and rather spasmodic tentacular concerts were induced. These did not appear to be focused in the direction of the prey, as they were with *D. pulex*, and were not accompanied by an opening of the mouth or any other feeding response. If *D. pulex* were then provided to the *Hydra* that already had *Scapholeberis* available as prey, the *Hydra* did not attempt to feed on the *Daphnia*. Thus, there appears to be an initial induction of a powerful tentacular concert, with a possible inhibition of the subsequent feeding response. *Scapholeberis* homogenate caused two types of responses: 1) immediate writhing followed by weak concerts with a higher frequency of concerts toward the end of the observation period, and 2) a few weak concerts and several whole body contractions.

When *Simocephalus* were provided to *Hydra* as prey items there was a very striking absence of response. *Simocephalus* adults can swim near and even among the tentacles and evoke no visible response, in much the same fashion as the clown fish can swim among the tentacles of the sea anemone. Occasionally, several nematocysts (desmonemes) will entangle an antenna of *Simocephalus*, but in a few seconds the cladoceran will be seen to shake off this tenuous attachment and swim away unharmed. When offered *D. pulex* subsequent to the *Simocephalus*, the *Hydra* quickly attacked the *Daphnia*. The homogenate of *Simocephalus*, as well as that of the remaining cladocerans studied, did evoke the feeding concert response in *Hydra* at a rate similar to that produced by the homogenate of *D. pulex*.

The pattern of concert behavior differed with *Simocephalus* in a marked manner. After introduction of the homogenate, there was frequently no response for the first minute of observation, followed by a burst of concerts, then a gradual decline in the rate of concerting. Thus, although intact, *Simocephalus*, particularly the adults, rarely elicit any attack by *Hydra*. There was no apparent inhibition of the glutathione-mediated feeding response when crushed prey were presented. This suggests that the differential response to the prey may be occurring at the site of nematocyst activation.

DISCUSSION

The results of our research indicate that both species of *Hydra* used in our investigations could conceivably be important predators in pond communities. Unlike other invertebrate predators, however, *Hydra* are not size-selective but appear to select prey on other characteristics. This result conflicts with Cuker and Mozley (1981) who found that *Hydra* in arctic ponds prefer small prey items, such as *Cyclops* species and *Bosmina*. The difference in results may be due to the inclusion in our study of other than limnetic cladocerans though Schroeder and Callaghan (1982) saw no evidence of prey selectivity among an array of limnetic cladocerans and copepods. In the present study limnetic zooplankton species, such as *Daphnia* and *Ceriodaphnia*, suffered high mortality regardless of size, while zooplankton species more often associated with the shallow littoral zone were not preyed upon extensively. Observations of behavior together with feeding trials suggest that those species living in close association with the *Hydra* seem to have evolved some means of defense.

The defense mechanisms of these cladocerans may include one or more of the following features: 1) an exoskeleton thick enough to prevent the penetration of *Hydra* nematocysts, specifically stenoteles; 2) an immunity to the toxin released from the nematocysts; 3) biochemical characteristics which could inhibit firing of the nematocysts (Lubbock, 1979), and 4) patterns of swimming behavior and location in the water column that result in different encounter and escape probabilities and hence varying vulnerabilities to predation. It should be emphasized that the evolution of any of these characteristics may not have been primarily in response to selection caused by *Hydra* but may be pre-adaptations.

All of these mechanisms may be present to some extent in *Simocephalus*. When nematocysts are fired at *Simocephalus*, as evidenced by the threads attaching the tentacles to the prey, the *Simocephalus* will often sit for a moment and then swim off, unharmed. This would suggest that the exoskeleton is not allowing penetration of the nematocysts or that the *Simocephalus* are immune to the toxin. The only other documented instance of apparent immunity to *Hydra* nematocysts is the chydorid cladoceran, *Anchistropus*, that actively preys on *Hydra* (Borg, 1935). But the observation that nematocysts are rarely fired even when the *Hydra* are in contact with this prey (*i.e.*, providing mechanical stimulation) suggests that the *Simocephalus* possess a cloaking mechanism such that the *Hydra* do not respond to their presence.

This is not to say that *Hydra* will not capture and feed on *Simocephalus*. Immature individuals, especially first instars, are readily preyed upon by larger *Hydra*. It may be that younger individuals have thinner carapaces, greater susceptibility to the toxin, or lack of strength to break from the threads of the nematocysts. In a mixed population, however, adults are usually ignored and the immature individuals taken only occasionally. *Daphnia* adults and juveniles are preyed upon to a much greater extent.

The nematocysts of several other coelenterates have been shown to have associated chemosensory cells (Mariscal and Bigger, 1976; Satterlie and Case, 1978) in addition to the mechanosensory cnidocils. Coelenterate nematocysts and/or sensory cells appear to be capable of distinguishing between and responding to surfaces of different chemical composition (Lubbock, 1979), even to the extent that inter-clonal differences of the anemone *Anthopleura elegantissima* can be recognized (Francis, 1973). Chemical substances associated with the carapace of *Simocephalus* may differ in some subtle manner from those of other cladoceran genera such that the response induced in the chemosensory cells of *Hydra* may be below a threshold level and nematocysts are not discharged. Enzyme inhibitors have been shown to reduce nematocyst discharge in *Hydra* in the presence of appropriate chemosensory stimuli (Lentz and Barnett, 1962; Lentz, 1966). Such inhibitors may be associated with the *Simocephalus* carapace surface, thereby preventing threshold levels of chemosensory stimulation from being reached.

The release of glutathione from wounded prey stimulates the feeding response in *Hydra* (Loomis, 1955). Any slowing of the response by the tentacles (which generally involves further entanglement of the prey and additional encounters with nematocysts) will allow increased escape time. Such a factor could be a substrate which competes with glutathione receptors and would account for the apparent delay in concert response with *Simocephalus* homogenate.

Though living in close proximity with *Hydra*, *Simocephalus* will only occasionally come in contact with the predator, as individuals spend much of their time attached to some substrate and swim only when disturbed. Clearly, this behavior decreases the number of encounters with *Hydra* and partially explains the results of the feeding trials. However, the small volume of the cups and large number of prey/cup ensured numerous opportunities for contact between predator and prey. Behavioral characteristics may also explain the reduced susceptibility of *Scapholeberis*. Individuals of this genus live in or near the surface film. Although *Hydra* have been observed attached to the roots of such floating vegetation as *Lemna*, and will float from the surface under adverse conditions (Lomnicki and Slobodkin, 1966), large areas of open water reduces the likelihood of *Scapholeberis* encountering *Hydra*.

Neither *D. pulex* nor *Ceriodaphnia* appear to have any defense against predation of *Hydra*. These two species are inhabitants of open water and would, therefore, encounter *Hydra* infrequently in the natural environment. Pressure to evolve a defense mechanism against *Hydra* may have been slight.

Community composition may thus be radically affected by the presence or absence of *Hydra*. *Daphnia* spp. were eliminated in our laboratory aquaria, but these have relatively large surface area:volume ratio such that *Hydra* were found on at least five of the six surfaces. The *Daphnia* thus had little room for error. In a natural environment the volume of open water may be considerably greater thereby reducing encounter frequency to a level permitting coexistence of *Hydra* and *Daphnia*. However, the cladoceran community would be expected to be dominated by *Simocephalus* and *Scapholeberis* regardless of volume of water where *Hydra* is in common abundance.

In conclusion, we have shown that *Hydra* feed differentially on commonly available cladoceran prey. The criteria for selection remain unknown, but it is clear that mechanisms protecting individuals exist in those potential prey species living in closest proximity to *Hydra*. We hope this initial research will lead to a better understanding of both *in situ* feeding ecology of *Hydra* and the role of "minor" predator groups in structuring pond zooplankton communities.

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2. ***Figures.*** Figures should be no larger than 8½ by 11 inches. The dimensions of the printed page, 5 by 7¾ inches, should be kept in mind in preparing figures for publication. We recommend that figures be about 1½ times the linear dimensions of the final printing desired, and that the ratio of the largest to the smallest letter or number and of the thickest to the thinnest line not exceed 1:1.5. Explanatory matter generally should be included in legends, although axes should always be identified on the illustration itself. Figures should be prepared for reproduction as either line cuts or halftones. Figures to be reproduced as line cuts should be unmounted glossy photographic reproductions or drawn in black ink on white paper, good-quality tracing cloth or plastic, or blue-lined coordinate paper. Those to be reproduced as halftones should be mounted on board, with both designating numbers or letters and scale bars affixed directly to the figures. All figures should be numbered in consecutive order, with no distinction between text and plate figures. The author's name and an arrow indicating orientation should appear on the reverse side of all figures.

3. ***Tables, footnotes, figure legends, etc.*** Authors should follow the style in a recent issue of *The Biological Bulletin* in preparing table headings, figure legends, and the like. Because of the high cost of setting tabular material in type, authors are asked to limit such material as much as possible. Tables,

Continued on Cover Three

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THE MARINE CERCARIAE OF THE WOODS HOLE, MASSACHUSETTS REGION, A REVIEW AND A REVISION

HORACE W. STUNKARD

The American Museum of Natural History, New York, and the Marine Biological Laboratory, Woods Hole, Massachusetts

INTRODUCTION

A survey of the marine cercariae of the Woods Hole, Massachusetts region was made by Stunkard (1970). That report was concerned with fifteen families and thirty-two species of digenetic trematodes. Their morphology, definitive and intermediate hosts, and systematic relations were considered. It was noted that the first intermediate host, the molluscan, is more specific than the definitive vertebrate host and that the second intermediate host, when present, may be a mere paratenic or transport host. In it, the metacercariae may encyst, or not, may fail to develop, or grow to almost full size and attain sexual maturity, a condition known as progenesis. The evolution of life-histories in the Digenea was discussed by Stunkard (1957a, 1959, 1975). Ecological relations and environmental conditions are more complex and diverse for marine trematodes than for terrestrial and freshwater species. The maintenance and experimental infection of intermediate and definitive hosts entail more difficulties with marine than with freshwater forms. Accordingly, the elucidation of life-histories of marine digenetic trematodes has not been a favored line of research. The description and naming of the sexually mature form of a species is a useful and praiseworthy task, but until the larval and developmental stages are known, the specific description is incomplete and may be faulty. For definitive classification and the determination of phylogenetic relations, it is essential that information of the complete life-history be available.

HISTORICAL BACKGROUND

In the early history of helminthology, specific descriptions were based on the morphology of the sexually mature stage of the parasite. But with the discovery of life-cycles, larval stages, asexual reproduction in intermediate hosts, biogeography, paleontology, genetic and parasitic specificity, descriptions are based on more complete information. In an address on "Parasitology in the world today," delivered at the opening plenary session of the Second International Congress of Parasitology, held in Washington, D. C., the late Jean G. Baer (1970) declared that sound taxonomy must remain the backbone of modern parasitology. He stated that parasitology is no longer a descriptive science of the pigeonhole type, but requires accurate information on the specific identity of both host and parasite, and it takes into account the ecology of the individual species, their physiology, life-cycles, genetic variation and even their social behavior.

The correlation of comparative anatomy, life-cycles, intermediate hosts, and systematic determination of digenetic trematodes is well illustrated by the blood-fluke parasites of birds and fishes. The avian species are dioecious and live in pairs in the mesenteric veins. They have long been known; Rudolphi (1819) described

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Distoma canaliculata from *Sterna galericulata*, collected in Brazil; a species that was named by Odhner (1912) as type of a new genus, *Ornithobilharzia*. Looss (1899) had erected the genus *Bilharziella* to receive *Distoma polonica* Kowalewski, 1895, a parasite of ducks, and Skrjabin and Zakharov (1920) erected the genus *Trichobilharzia* with *T. ocellata* (La Valette, 1855) Brumpt, 1931, from shore-birds as type. The specific name was based on *Cercaria ocellata* La Valette. The schistosomes are the only dioecious trematodes, and they occur only in the veins of birds and mammals. It is generally agreed that they were derived from monoecious spirorchid species parasitic in the arteries of their reptilian progenitors. The blood-flukes of existing reptiles are hermaphroditic and occur only in the arterial system. Stunkard (1923) discussed the origin of the dioecious condition and suggested that the constant higher temperature and increased oxygen content of the blood of birds and mammals may have been factors in shifting the worms from the arteries of reptiles to the veins of birds and mammals.

Cort (1928a) reported that *Cercaria elvae* Miller, 1923, from *Lymnaea marginata angulata* Michigan (identified by McMullen and Beaver, 1945, as a possible synonym of *C. ocellata*) produced severe dermatitis in man. He (1928b) stated that four of the five schistosome cercariae from the Douglas Lake area of Michigan produced a dermatitis on contact with human skin. Vogel (1930) described cercarial dermatitis in Germany. All of these cercariae are larvae of avian schistosomes that penetrate the delicate skin of their hosts to reach the blood vessels. In mammals, the skin is thicker, and they are unable to penetrate, and on repeated exposure, human beings develop immunity which results in the dermatitis, an allergic response.

In a broad report, Johnston (1917) described avian blood flukes, *Austrobilharzia terregalensis* from seagulls, *Larus novae-hollandiae*, in Australia. Chapin (1924) reported schistosome trematodes from ducks, *Marila affinis* taken in Maryland. The worms were assigned to the genus *Ornithobilharzia* Odhner, 1912, without specific designation, and deposited in the U. S. National Museum. When Price (1929) formulated his synopsis of the trematode family Schistosomatidae Looss, 1899, he studied the specimens of Chapin. He noted their resemblance to *Austrobilharzia terregalensis* Johnston, 1917, but also certain differences. Because of these differences, the Chapin's specimens were described as members of a new genus, *Microbilharzia*, with the specific name, *chapini*, in honor of their discoverer. No life-cycles or larval stages were known, and the limited information precluded any decision on relationships between the genera.

The cercarial dermatitis described by Cort (1928a, b), Vogel (1930), Talbot (1936), and later American authors was caused by schistosome larvae from freshwater snails. From 1934 to 1942, cercarial dermatitis was reported from the Lake district of central southern Canada in papers by McLeod. Four cercarial species were identified as the inciting agents: *C. elvae* Miller, 1923, *C. stagnicolae* Talbot 1936, *C. physellae* Talbot, 1936, and *Cercaria dermolestes* McLeod, 1936. The earlier studies were reviewed by McLeod and Little (1942) who reported that *C. physellae* Talbot, 1936, is the larva of *Pseudobilharzia querquedulae* McLeod, 1937.

Penner (1950) described a dermatitis-producing cercaria, *Cercaria littorinae* n. sp., from *Littorina planaxis* Philippi, a marine, operculate gastropod which is common on the rocky shores of southern California. It was compared to *Cercaria polonica* Szidat, 1929 and *Cercaria gyrauli* Brackett, 1940, but no further taxonomic consideration was attempted. This was the first report of cercarial dermatitis caused by a marine cercaria.

An epidemic of "swimmer's itch" in Narragansett Bay, Rhode Island was investigated by Stunkard and Hinchliffe (1952) who found that the dermatitis was caused by the schistosome *Cercaria variglandis* Miller and Northup, 1926, emerging from *Nassarius obsoletus* (Say). Adult worms were obtained by exposing various birds, nestling gulls, pigeons, and canaries to the larvae. Cort (1921) had demonstrated that in the schistosomes, sex is determined at fertilization and that all the progeny from a miracidium are the same sex. So, in order to secure both males and females in experimental infections, the birds were exposed to pooled cercariae from more than one snail. The adult worms were identified as *Microbilharzia chapini* Price, 1929, but since the name of the cercaria, *variglandis*, antedated that of the adult stage, *chapini*, the species was described as *Microbilharzia variglandis* (Miller and Northup, 1926) Stunkard and Hinchliffe, 1951. The new terminology was proposed in an abstract, Stunkard and Hinchliffe, (1951).

In an abstract, without supporting data, Penner (1953c) suppressed *Microbilharzia* Price, 1929, as a synonym of *Austrobilharzia* Johnston, 1917, and transferred the species *variglandis* to *Austrobilharzia*.

Chu (1952) reported cercarial dermatitis in Hawaii and suggested that it may have been caused by cercariae from the marine snail, *Littorina pintade* Wood, which was abundant on the bird islands near Oahu, Hawaii. Chu and Cutress (1954) exposed young noddy and sooty terns (*Anous stolidus pileatus* Scopoli and *Sterna fuscuta oahuensis*), chickens, and ducks to these cercariae and recovered adult schistosomes from the mesenteric veins of all these species. Natural infection by this fluke was found in ruddy turnstones, *Arenaria interpres interpres* (L.), killed on the island of Oahu. The Hawaiian specimens agreed closely with *Microbilharzia variglandis* as described by Stunkard and Hinchliffe (1952). However, distinct differences were noted between the males and the diagnosis of *Microbilharzia*. The differences appeared to be of specific value and to constitute a distinction between the species *chapini* and *variglandis*. They noted that Penner (1953c) had suppressed *Microbilharzia* Price, 1929, as a synonym of *Austrobilharzia* and transferred the species *variglandis* to *Austrobilharzia*. The morphological agreement between *Austrobilharzia variglandis* and the Hawaiian species was so complete that specific identity was apparent.

Bearup (1955) reported cercarial dermatitis in New South Wales, Australia, and experimental infections proved it was caused by a schistosome larva from *Pyrazus australis* Quoy and Gaimard. The situation closely paralleled that reported by Stunkard and Hinchliffe (1952); the larvae were very similar, and the Australian form was tentatively named *Cercaria variglandis* (Miller and Northup) subspecies *pyrazi* n. subspecies. Bearup (1956) reported that experimental infection of gulls and other birds proved that the cercaria from *Pyrazus australis* is the larval stage of *Austrobilharzia terregalensis* Johnston, 1917. The description of Johnston was revised and extended, based on an abundance of new material. Bearup did not cite the paper by Chu and Cutress (1954), and it is obvious that he had not seen it. Chu and Cutress (1954) had described the eggs of *A. variglandis* and the description of the eggs of *A. terregalensis* was similar, even to the small terminal spine, usually bent as in the letter "J." The more complete description of *A. terregalensis* agrees so consistently with the description of the Hawaiian species that their identity is obvious.

Short and Holliman (1961) described a schistosome cercaria from *Cerithidea scalariformis* taken in Apalachee Bay on the north shore of the Gulf of Mexico. Exposure of chicks and parakeets yielded adults, described as *Austrobilharzia penneri* n. sp. These larvae are similar and may be identical to *Cercaria Caribbea XLIX*, described by Cable (1956) from *Cerithidea costate* in Lucia Bay, Cabo Rojo, Puerto Rico.

The infection of shore-birds by schistosomes is almost universal; some ten genera are involved. There is little specificity; the same species may infect birds belonging to diverse families. Cercarial dermatitis is a one to three week annoyance to bathers, clam diggers, workers in rice paddies and other marine and freshwater installations. It is trivial in comparison with schistosomiasis, one of the most serious and most extensive of human infections. In its 1982 report, the World Health Organization estimated more than two-hundred million cases, mostly in tropical areas of South America, Africa, the Middle East, and Asia, especially China, Japan, and Indonesia.

The problems encountered in the classification and taxonomy of the schistosomes of birds are met with even more difficult situations in the blood flukes of fishes. Two families are recognized; Sanguinicolidae Graff, 1907 and Aporocotylidae Odhner, 1912, but the diagnostic characters are uncertain, indefinite and often inconsistent. They occur in both marine and freshwater hosts, and there is little correlation between hosts and parasites. In certain species the cercariae develop in sporocysts, in others in rediae; some in the digestive gland and gonads of snails or bivalve mollusks; others in the coelomic cavities of polychaete annelids. There are cercariae with long, brevifurcate tails, long tails without furcae, and others with greatly reduced, stumpy tails.

Three species have been described from the Woods Hole region. Linton (1915a) described sporocysts and cercariae from the coelomic cavities of the polychaete annelid, *Hydroides dianthus* (Verrill). The sporocysts were oval, often attenuated at one or both ends; the cercariae were furcocercous with short furcae, and with finfolds on the middorsal surface of the body, and on the furcae. In the same issue of the *Biological Bulletin,* Linton (1915b) described similar sporocysts and cercariae from the scallop, *Pecten irradians.* The cercariae were slender, with tails longer than the body, and according to the figure, brevifurcate. Linton observed movements of the body and especially the anterior end that "suggest adaptations to enable the cercariae to penetrate soft membranes of their secondary host." He noted that the cercariae from *Hydroides dianthus* and *Pecten irradians* resemble *Cercaria cristata* La Valette. Martin (1944b) described a new species, *Cercaria solenomyae* listed as a probable blood fluke, from the bivalve mollusk, *Solemya velum.* The cercariae developed in spherical to oval sporocysts in the digestive gland and gonad of the clam. The cercariae had the diagnostic features of the blood-flukes of fishes, except for short, stumpy tails. This species has not been cited in the literature since the original description and its taxonomic relations are obscure.

The species from *H. dianthus* [= *Eupomatus dianthus* (Verrill) Morch, 1863, according to Hartmann in Martin (1952)] was named *Cercaria loossi* by Stunkard (1929) and further data were provided by Martin (1944a) and Rankin (1946). The species from *Pecten irradians* is still unnamed, and for it I propose the name *Cercaria martini* n. sp., in recognition of the contributions by Dr. Martin to knowledge of the digenetic trematodes. The report of a cercaria from an annelid was surprising, since all previously described cercariae were found in mollusks. However, two additional species have been described from annelids. Martin (1952) described cercariae developing in rediae in the terrebellid annelid, *Lanicides vayssierei,* taken in dredges by the U. S. Navy Antarctic Expeditions of 1947–1948. The annelids were submitted to Dr. Olga Hartmann for identification, and Martin described the rediae and cercariae from the coelomic cavities of the worms as *Cercaria hartmannae* n. sp. A third species of cercariae developing in polychaete annelids was discovered by Oglesby (1961). He described *Cercaria amphictereis* n. sp., from *Amphictereis gunneri floridus* Hartmann, taken in Florida. The cercariae were produced in rediae, and the tail was non-furcate and slightly shorter than the body.

The cercariae of blood-flukes of fishes have been compared with *Cercaria cristata* La Valette, 1855, a brevifurcate larva with finfolds on the middorsal surface of the body and on the furcae. But the species was from *Lymnaea stagnalis* (L.), a freshwater snail; it is not clearly differentiated, and Ejsmont (1926) observed that it had been described from four different snails and probably comprised more than one species. The descriptions of the blood-flukes of fishes, their morphology, and developmental stages provide no satisfactory taxonomic arrangement. Martin (1944a) assigned *Cercaria loossi* to *Aporocotyle* and the determination has been generally approved. *Cercaria solemyae* Martin, 1944b agrees substantially with the cercariae of *Sanguicola inermis* and *S. armata* as described by Scheuring (1922) and Ejsmont (1926) and is included in the Sanguinicolidae.

MARINE TREMATODES OF THE WOODS HOLE REGION

Families, genera, and species

- ACANTHOCOLPIDAE Lühe, 1909.
  - *Stephanostomum tenue* (Linton, 1898) Linton, 1934.
  - *Stephanostomum dentatum* (Linton, 1900) Linton, 1940.
- APOROCOTYLIDAE Odhner, 1912.
  - *Cercaria loossi* Stunkard, 1929.
- BUCEPHALIDAE Poche, 1907.
  - *Rhipidocotyle transversale* Chandler, 1935.
  - *Rhipidocotyle lintoni* Hopkins, 1954.
- CRYPTOGONIMIDAE Ciurea, 1933.
  - *Siphodera vinaledwardsi* (Linton, 1901) Linton, 1910.
- DEROPRISTIDAE Skrjabin, 1958.
  - *Deropristis inflata* (Molin, 1859) Odhner, 1902.
- DIPLOSTOMATIDAE Poirier, 1886.
  - *Diplostomum nassa* (Martin, 1945) Stunkard, 1973.
- ECHINOSTOMATIDAE Poche, 1926.
  - *Himasthla quissetensis* (Miller and Northup, 1926) Stunkard, 1938.
  - *Himasthla compacta* Stunkard, 1960.
  - *Himasthla littorinae* Stunkard, 1966.
- FELLODISTOMATIDAE Nicoll, 1913.
  - *Lintonium vibex* (Linton, 1900) Stunkard and Nigrelli, 1930.
  - *Proctoeces maculatus* (Looss, 1901) Odhner, 1911.
- GYMNOPHALLIDAE Dollfus, 1939.
  - *Cercaria myae* Uzmann, 1952.
  - *Parvatrema borealis* Stunkard and Uzmann, 1958.
- HEMIURIDAE Lühe, 1901.
  - *Lecithaster confusus* Odhner, 1905.
  - *Tubulovesicula pinguis* (Linton, 1940) Manter, 1947 (syn. *Distomum rufoviride* Rudolphi of Linton, 1898).
- HETEROPHYIDAE Leiper, 1909.
  - *Cryptocotyle lingua* (Creplin, 1825) Fischoeder, 1903.
- HOMALOMETRIDAE Cable and Hunninen, 1942.
  - *Homalometron pallidum* Stafford, 1904.
- LEPOCREADIIDAE Odhner, 1905.
  - *Neopechona pyriforme* (Linton, 1900) Stunkard, 1969.
  - *Neopechona cablei* Stunkard, 1980.

Lepocreadium areolatum (Linton, 1900) Stunkard, 1969.
Lepocreadium setiferoides (Miller and Northup, 1926) Martin, 1938.
MICROPHALLIDAE Ward, 1901.
Spelotrema simile (Jägerskiöld, 1900) Looss, 1902.
Spelotrema nicolli Cable and Hunninen, 1938.
Atriophallophorus minutus (Price, 1934) Deblock and Rosé, 1964.
Gynaecotyla adunca (Linton, 1905) Yamaguti, 1939.
Microphallus limuli Stunkard, 1951.
Odhneria odhneri Travassos, 1921.
MONORCHIIDAE Odhner, 1911.
Monorcheides cumingiae (Martin, 1938) Martin, 1940.
Asymphylodora amnicolae Stunkard, 1959.
Lasiotocus minutus (Manter, 1931) Thomas, 1959.
Lasiotocus elongatus (Manter, 1931) Thomas, 1959.
NOTOCOTYLIDAE Lühe, 1909.
Notocotylus minutus Stunkard, 1960.
Notocotylus atlanticus Stunkard, 1966.
Paramonostomum alveatum (Mehlis in Creplin, 1846) Lühe, 1909.
Paramonostomum parvum Stunkard and Dunihue, 1931.
Uniserialis breviserialis Stunkard, 1967.
OPECOELIIDAE Ozaki, 1925.
Podocotyle atomon (Rudolphi, 1802) Odhner, 1905.
Opecoeloides vitellosus (Linton, 1900) von Wicklen, 1946.
Syn. *Distomum vitellosum* Linton, 1900; *Cymbephallus vitellosus* (Linton, 1900) Linton, 1934; *Anisoporus manteri* Hunninen and Cable, 1940.
PHILOPHTHALMIDAE Travassos, 1918.
Parorchis avitus Linton, 1914.
Syn. *Cercaria sensifera* Stunkard and Shaw, 1931; *Parorchis acanthus* Cable and Martin, 1935.
RENICOLIDAE Dollfus, 1939.
Renicola thaidus Stunkard, 1964.
SCHISTOSOMATIDAE Looss, 1899.
Austrobilharzia variglandis (Miller and Northup, 1926) Penner, 1953.
SANGUINICOLA Graff, 1907.
Cercaria solemyae Martin, 1944.
Cercaria martini n. sp.
ZOOGONIDAE Odhner, 1911.
Zoogonus rubellus (Olsson, 1867) Odhner, 1902.
Zoogonoides laevis Linton, 1940.

LARVAL TREMATODES OF THE WOODS HOLE AREA

**Designates publication with figures of asexual generations*

In *AMNICOLA LIMOSA:*

1. Cercaria of *Asymphylodora amnicolae* Stunkard, 1959; distomate, tailless; acetabulum larger than oral sucker, situated at or slightly posterior to midbody; penetration glands lateral, preacetabular; genital organs represented by germinal cells, dorsal and posterior to acetabulum; excretory vesicle saccate; system stenostomate, flame-cell formula 2 [(4 + 4 + 4 + 4) + (4 + 4 + 4 + 4)]. Sporocysts obtained by feeding embryonated eggs to laboratory reared snails, *A. limosa.* Rediae

simple; sausage shaped; without collar or feet. Cercariae emerge from rediae, migrate to tentacles of snail, attach by acetabulum with both ends free; may not penetrate same snail but transfer to an uninfected one; may encyst or remain unencysted, but grow, become mature and gravid. Eggs from snails were used to infect juvenile snails. Adults in natural and experimental infections of *Fundulus diaphanus* and *Perca flavescens.* Life-cycle confirmed by Larson (1961). *(Stunkard, 1959).

In *ANACHIS AVARA* SAY:

1. Cercaria of *Neopechona pyriforme* (Linton, 1900) Stunkard, 1969; distomate; ocellate; trichocercous; integument spinose; tail with 22 pairs of lateral finlets; eight pairs of penetration glands; acetabulum near midbody; ceca long, open into the excretory bladder; vesicle cylindrical, contains concretions, system stenostomate, flame-cell formula 2 [(4 + 4 + 4) + (4 + 4 + 4)]; eggs embryonated, 8–10 days, released miracidia which penetrated juvenile *A. avara,* transformed into sporocysts and produced rediae in 5–6 weeks; rediae with attenuate, curved tail-like posterior ends; metacercariae unencysted in *Mnemiopsis leidyi* and medusae of *Bougainvillia carolinensis, Gonionemus vertens, Chrysaora quinquecirrha, Pelagia noctulica,* and *Aequoria forskalea;* adults experimentally; in scup, *Stenotomus chrysops.* Species described by Linton (1901) as *Distoma pyriforme* from several species of fish. *(Stunkard, 1969).

In *BITTIUM ALTERNATUM* (SAY):

1. Cercaria of *Spelotrema nicolli* Cable and Hunninen, 1938. [Listed as *Microphallus nicolli* in Stunkard (1970).] Produced in oval to elongate sporocysts; cercaria small, monostomate, with simple stylet, non-oculate, simple tail about as long as the body, with fine cuticular annulations; oral sucker only developed part of digestive system; four pairs of penetration glands, ducts open at the stylet; excretory vesicle with two lateral lobes, collecting ducts mesostomate, flame-cell formula 2 [(1 + 1) + (1 + 1)]. Penetrate the gills of blue crab, *Callinectes sapidus,* migrate by vascular system to all parts of body. Metacercariae encysted, outer wall thick, inner wall membranous; genital primordia anterior to excretory vesicle; flame-cell formula 2 [(2 + 2) + (2 + 2)]. Adults experimentally in gulls, naturally in shore-birds. *(Cable and Hunninen, 1940).
2. Cercaria of *Siphodera vinaledwardsi* (Linton, 1901) Linton, 1910. Monostomate; ocellate; pleurolophocercous; integument spined anterior to the ocelli; tail long, bearing dorsal and ventral finfolds; excretory system mesostomate, with Y-shaped vesicle and flame-cell formula 2 [(2 + 2) + (2 + 2)]; seven pairs of penetration glands. Develop in saccate to elongate rediae, metacercariae encysted in flounders, especially *Paralichthys dentatus;* adults experimentally in toadfish, *Opsanus tau.* Species described by Linton, (1901) as *Monostomum vinaledwardsi.* *(Cable and Hunninen, 1942a).
3. Cercaria of *Deropristis inflata* (Molin, 1859) Odhner, 1902. At least two generations of clavate to cylindrical rediae; cercaria distomate, ocellate, modified trichocercous; tail shorter than body with median, ventral finfold and with rows of tubercules on each side; eight pairs of penetration glands, situated at the sides of the esophagus, ducts in two bundles on both sides of the eye-spots; excretory vesicle saccate, thick walled, collecting ducts mesostomate, flame-cell formula 2 [(3 + 5 + 8) + (3 + 3 + 5)], but not yet complete; metacercariae in *Nereis virens,* adults experimentally in eels, *Anguilla rostrata.* *(Cable and Hunninen, 1942b).

In *CUMINGIA TELLINOIDES* (CONRAD) and *TELLINA TENERA* (SAY):

1. Cercaria of *Monorcheides cumingiae* Martin, 1938, 1940. Sporocysts in *Cumingia tellinoides*, saccate to cylindrical; cercariae oval, distomate, spinose on anterior half of body; ocellate; oral sucker subterminal with three circles of papillae; two pairs of penetration glands; pharynx bulbous; ceca short, not extending back of the acetabulum; excretory vesicle saccate, tubules mesostomate, flame-cell formula 2 [(2 + 2) + (2 + 2)]. Tail with lateral cup-shaped lappets. Metacercariae encysted in foot and siphon of *Cumingia tellinoides* and *T. tenera;* adults experimentally in eels and flounders. *(Martin, 1938; life-cycle Martin, [1940]).

In *GEMMA GEMMA:*

1. Cercaria of *Parvatrema borealis* Stunkard and Uzmann, 1958. Sporocysts oval to cylindrical, in interlobular spaces of digestive gland, birth pore at the apical end, small individuals motile, large ones inactive. Cercaria distomate, furcocercous, oral sucker with small, ear-like projections, tail stem shorter than body, furcae about as long as stem; no ocelli, oral sucker and acetabulum about same size, digestive ceca preacetabular; excretory vesicle saccate with dorsal pouch and arms that extend to the pharyngeal level, and contain concretions. Metacercariae unencysted, in the mantle, spinose; excretory vesicle V-shaped, flame-cell formula 2 [(2 + 2) + (2 + 2)]; adults in eider ducklings, *Somateria mollissima.* *(Stunkard and Uzmann, 1958).
2. Cercaria of *Lasiotocus minutus* (Manter, 1931) Thomas, 1959. This cercaria was described as a new species, *Cercaria adranocerca;* by Stunkard and Uzmann (1958). The specimens were found in the Boothbay Harbor area of Maine. They were found later at Woods Hole and further study proved them to be a stage in the life-cycle of *L. minutus.* Adult worms from *Menidia menidia* taken at Beaufort, North Carolina were described by Manter (1931) as *Genolopa minuta.* The status and validity of *Lasiotocus* Looss, 1907, *Genolopa* Linton, 1910, and *Proctotrema* Odhner, 1911, are equivocal. The cercariae are distomate; micro-cercous, without stylets or ocelli. They are not liberated from the clam but encyst in the mollusk and are discharged singly or in masses. In encystment, the secretion forms a large, loose, flexible membranous sac, the distal tip of which is adhesive. So when released from the clam, the metacercariae may form large clusters. The excretory vesicle is saccate, the system mesostomate, the flame-cell formula 2 [(2 + 2) + (2 + 2)]; adults in *Menidia menidia.* *(Stunkard, 1981a).
3. Cercaria of *Lasiotocus elongatus* (Manter, 1931) Thomas, 1959. The species was described but not named by Linton (1901) from *M. menidia,* with two figures. Linton (1905) reported it from *M. menidia* at Beaufort, North Carolina. Manter (1931) did not refer to Linton's (1901) report and the specimens described by Linton (1905) were assigned to *Genolopa minuta.* The species was described by Manter (1931) as *Genolopa elongatus* n. sp.. Sporocysts occurred in the digestive gland and gonad of the clam. After successive generations of sporocysts, cercariae were produced. Sporocysts oval to elongate, with a basal portion and anterior extension with the birth pore. Cercaria distomate, acetabulum near midbody, larger than oral sucker, integument spined, tail microcercous, digestive ceca long, body wall underlaid with rows of cystogenous cells, no stylet or ocelli. Reproductive organs represented by a column of germinal cells, postacetabular; excretory vesicle saccate, lined with epithelium, when filled may extend almost to acetabulum, no concretions, flame-cell formula 2 [(2 + 2) + (2 + 2)]; cercariae encysted in clam, liberated into sea water, through the gills; cyst thick walled, partially enclosed in an unformed

narrow coat of jelly-like material that adhered lightly to vegetation, adults in *Menidia menidia, Fundulus heteroclitus* and *Pseudopleuronectes americanus.* *(Stunkard, 1981b).

In *HYDROBIA MINUTA:*

1. Cercaria of *Himasthla compacta* Stunkard, 1960. At least two generations of rediae with small collars and feet that are used in locomotion. Cercariae small, few, leave the redia early and mature in haemocoele; cercaria distomate, with reniform collar bearing 29 spines, 25 in a lineal row and 2 on each side below and between the ends of the row; integument with imbricate spines; acetabulum near midbody, larger than oral sucker; esophagus long, ceca long, five pairs of penetration glands; tail simple with excretory pores on sides; excretory vesicle saccate, collecting ducts stenostomate, may have concretions; flame-cell formula unknown. Cercariae encyst in *Mya arenaria* and other bivalves; adults experimentally in *Larus argentatus.* *(Stunkard, 1960b).

2. Cercaria of *Atriophallophorus minutus* (Price, 1934) Deblock and Rosé, 1965. Listed as *Levinseniella minuta* Price, 1934 in Stunkard (1970). Cercariae develop in second generation sporocysts, leave the sporocyst while immature and encyst in the haemocoele of snail. There is no second intermediate host. Cercaria monostomate, tailless, integument without spines; encyst early; metacercariae developed to maturity in white mice and hamsters, but do not persist. Adults were obtained from scaup ducks, *Nyroca affinis.* Eggs from worms developed in mice, were fed to *H. minuta* which became infected and after two months contained encysted metacercariae. The species is obviously a parasite of birds. Deblock and Rosé (1965) erected the new genus *Atriophallophorus* for this species and Yamaguti (1975) approved. Burns (1963) reported what may be the same species in the snail, *Oxytrema silicula,* in Oregon. *(Stunkard, 1958).

3. Cercaria of *Homalometron pallidum* Stafford, 1904. Adult worms from *Fundulus heteroclitus,* eggs were embryonated, fed to *H. minuta,* and 6 weeks later snails were liberating cercariae identical with those of natural infections. Cercaria oval to elongate, in daughter rediae; emerge at night; body oval to elongate; tail about length of body, with three pairs of papillae bearing setae and a low dorsoventral finfold; oral sucker with circumoral spines; acetabulum postequatorial; paired ocelli; ceca long; 6 pairs of penetration glands in two groups anterolateral to acetabulum; excretory vesicle saccate, wall glandular, flame-cell formula $2\ [(3 + 3 + 3) + (3 + 3 + 3)]$; metacercariae encysted in *Gemma gemma* and small polychaete annelids; metacercariae from *G. gemma* fed to *Fundulus* spp. and developing stages recovered. *(Stunkard, 1964a).

4. Cercaria of *Notocyotylus minutus* Stunkard, 1960a. Adults, described by Stunkard and Dunihue (1931) as *Notocotylus gibbus* (Mehlis in Creplin, 1846); based on the account of Kossack (1911). But *N. gibbus* was redescribed by Szidat (1935) and morphologically is quite different from the specimens of Stunkard and Dunihue. Also, worms were referred by Harwood (1939) to *N. imbricata* (Looss, 1893) Szidat, 1935, but *Cercaria imbricata* Looss, 1893 was later named as the larva of *N. attenuatus* Rudolphi, 1809. In rediae, oval to clavate; cercaria few, large, monostomate, ocellate, anterior region of body pigmented as eye-spots disintegrate; no penetration glands; cystogenous glands extensive; excretory vesicle saccate, ducts stenostomate, fuse at the level of the eye-spots with a median anterior loop, contain concretions; common duct from bladder extends into the tail with pores on the sides. After short swimming period encysts on solid objects; cysts circular, 0.12–0.16

mm in diameter; metacercaria do not grow but remain infective for long periods, fed to eider ducklings, developed to adults; morphologically identical with specimens of Stunkard and Dunihue. *(Stunkard, 1960a).

5. Cercaria of *Spelotrema limuli* (Stunkard, 1951) Yamaguti, 1971. (Described as *Microphallus limuli* in Stunkard (1970)). Successive generations of sporocysts; cercaria oval, monostomate, with simple stylet; oral sucker only developed part of digestive system; integument with scale-like spines, tail simple, about body length; 4 pairs of penetration glands; V-shaped excretory bladder, mesostostonate ducts, flame-cell formula 2 [(1 + 1) + (1 + 1)]; young horseshoe crabs exposed in bowls with cercariae yielded heavy infections. Metacercarial cysts in all organs and tissues, wall thick, hyaline, composed of two layers; reproductive organs well developed; adults in white mice, but lost after a few days; natural hosts probably birds. *(Stunkard, 1968).

In *HYDROBIA SALSA:*

1. Cercaria of *Notocytylus atlanticus* Stunkard, 1966. Redia large, orange-yellow, esophagus as long as pharynx, ceca half body length; no locomotor appendages, birth pore ventral at pharyngeal level, excretory pores lateral, posterior to midbody, flame cell formula 2 [(1 + 1) + (1 + 1)]. Cercaria with parallel sides, monostomate, trioculate, with locomotor appendages at posterolateral corners of body, diffuse pigment in anterior third of body as eye-spots disintegrate; tail slender, with excretory pores on sides, flame cell formula 2 [(1 + 1 + 1) + (1 + 1 + 1)]; Cercaria emerge about midday, encyst at once if irritated, otherwise after swimming for 2–3 hours; cyst flat, circular, average 0.18 mm in outer, 0.16 mm inner diameter; larvae do not develop, infective at once, persist for weeks; adults after feeding metacercariae to eider ducklings. *(Stunkard, 1966b).

2. Cercaria of *Notocotylus minutus.* (See *Hydrobia minutus*).

3. Cercaria of *Paramonostomum alveatum* (Mehlis in Creplin, 1846) *Lühe,* 1909. Redia large, may be 1 mm in length, golden yellow color; cercaria trioculate, emerge while immature, completes development in haemal sinuses of snail, emerge principally about midday, body oval to ovate, average 0.40 by 0.15 mm, convex dorsally, concave ventrally, tail simple, same length as body, when body is contracted tail is extended and *vice versa,* postero-lateral corners of body with reversible and retractile locomotor appendages, cystogenous cells with rhabites, ceca long, medial to excretory ducts, end blindly; excretory vesicle saccate, collecting ducts form ring under esophagus, contains concretions, excretory tube into tail, pores lateral; metacercariae encysted on shell and other hard objects, adults experimentally in chicks, domestic and eider ducklings and gulls, 0.5–0.85 by 0.04–0.5 mm eggs with long polar filaments. *(Zelikman, 1966; Stunkard, 1967a).

4. Cercaria of *Paramonostomum parvum* Stunkard and Dunihue, 1931. Redia and daughters migrate actively, cercariae emerge from parent while still immature; cercaria monostomate, triocellate, ovate, smaller than *P. alveatum,* average 0.30 by 0.10 mm, tail simple, about body length, cystogenous cells in a layer from anterior to posterior of body. Excretory vesicle saccate, ring passes posterior to ocelli, contains concretions; recurrent tubule passes posteriad from the anterolateral face of ring, near midbody divides into anterior and posterior branches, formula probably 2 [(3 + 3 + 3) + (3 + 3 + 3)]; metacercariae in circular cysts 0.13–0.14 mm in diameter; adults obtained after feeding metacercariae to eider ducklings, 0.25–0.50 by 0.2–0.3 mm, minute spines on ventral surface. *(Stunkard, 1967a).

5. *Uniserialis breviserialis* Stunkard, 1967. Redia oval to elongate, motile, with-

out feet, older orange-yellow, up to 1 mm. Intestinal ceca long, birth pore ventral at esophagus; 2 flame cells one in anterior, other in posterior part of body, pores lateral, near midbody. Cercaria oval, monostomate, with posterolateral locomotor appendages, triocellate; tail shorter than body; esophagus long, ceca long, end blindly; emerge about midday, encyst after 2–3 hours; cyst circular, average 0.195 mm outer diameter, 0.175 mm inner diameter; worms do not grow; no secondary intermediate host; mature in domestic ducklings and day old chicks. *(Stunkard, 1967c).

In *HYDROIDES DIANTHUS* (VERRILL, 1873):

1. *Cercaria loossi* Stunkard, 1929. Produced in orange colored sporocysts in the coelomic cavities of the posterior two-thirds of the annelid; emerge through the genital ducts. Cercaria brevifurcocercous, with middorsal finfold, integument spined anteriorly, otherwise smooth, penetrating organ average 35 μ long, with several nuclei, five pairs of penetration glands; excretory vesicle small, V-shaped, with tubule in the tail stem, bifurcated; in furcae with pores at the tips. *(Linton, 1915a; Martin, 1944a; Rankin, 1946).

In *LAEVICARDIUM MORTONI:*

1. Cercaria of *Lintonium vibex* (Linton, 1900) Stunkard and Nigrelli, 1930. This cercaria was described by Martin (1945) as *Cercaria laevicardium* and by Cable (1954) as *Cercaria laevicardii.* Successive generations of elongate sporocysts occur in the digestive gland of *L. mortoni.* Cercaria oval, distomate, without stylet or ocelli; integument without spines; suckers about same size; acetabulum at midbody; ceca short, not postacetabular; tail trichocercous with 28 pairs of setae arranged in bands, aligned dorso-ventrally on opposite sides of the tail, designated "finlets" by Cable; 16 penetration glands; testes opposite, immediately postacetabular; ovary between testes; excretory vesicle V-shaped, arms extend to pharyngeal level, contain concretions; flame-cell formula 2 [(3 + 3) + (3 + 3)]; common stem extends through the tail, bifurcating at the end to form two excretory pores. Emerge in day and night, swim for about 12 hours, attach by tip of tail; are picked up by ctenophores, *Mnemiopsis leidyi,* migrate to aboral region; metacercariae grow to 3–4 times cercarial size; fed to small puffers, *Spheroides* maculatus, grew till end of summer. *(Martin, 1945; Cable, 1954; Stunkard, 1978).

In *LITTORINA LITTOREA* (Linn, 1758) Férussac, 1822:

1. Cercaria of *Cryptocotyle lingua* (Creplin, 1825) Fischoeder, 1903. Develop in rediae in digestive gland; named *Cercaria lophocerca* Lebour; Cercaria ovate to elongate; acetabulum one-third body length from anterior end; monostomate; ocellate; pleurolophocercous; emerge through gills; accumulate on light side of bowl; tail with dorsal finfold; oral sucker with acciculate spines, 18 pairs of penetration glands; basophilic cystogenous glands; excretory vesicle Y-shaped, flame-cell formula 2 [(3 + 7 + 7) + (7 + 7 + 7 + 7)]; penetrate skin of fishes, especially cunners, *Tautolabrus adspersus,* encyst in skin; metacercariae enclosed in pigment cells. Adults naturally in birds, gulls and terns, experimentally in dogs, cats, and rats, but do not persist. *(Stunkard, 1930).

2. *Cercaria parvicaudata* Stunkard and Shaw, 1931. In orange colored daughter sporocysts which form a mass in the wall of the digestive gland; oval to clavate, 0.03–0.09 by 0.03–0.12 mm with apical birthpore; cercaria oval to ovate, 0.14–0.36

by 0.05–0.12 mm, distomate; suckers of approximately same size, acetabulum in posterior half of body; esophagus long, ceca short; small stylet with 3 anterior points, 6 pairs of penetration glands; body filled with cystogenous gland cells; excretory vesicle Y-shaped; flame-cell formula 2 [(3 + 3 + 3) + (3 + 3 + 3)]; cercariae penetrate and encyst in parapodia of annelids, in various species of *Littorina* and in polyclad turbellarians. Adults unknown, probably renal parasites of birds. *(Stunkard, 1950).

In *LITTORINA OBTUSATA:*

1. Cercaria of *C. lingua.*
2. *Cercaria parvicaudata.*
3. Cercaria of *Himasthla littorinae* Stunkard, 1966. Repeated redial generations; young specimens with collar and locomotor appendages; birth-pore ventral, posterior to esophagus. Cercaria leaves redia in immature condition; body oval to ovate, distomate; reniform collar bearing 29 spines, acetabulum post-equatorial; esophagus long; ceca long; end blindly; 3 pairs of penetration glands; cystogenous glands fill body from end to end, excretory vesicle saccate, flame-cell formula 2 [(5 + 5 + 5 + 5 + 5) + (5 + 5 + 5 + 5 + 5)]. Emerge in both day and night, no response to light; swim at all levels; encyst in various mollusks, including snail from which they emerged; adults in domestic ducklings and laboratory raised gulls. *(Stunkard, 1966a).
4. Cercaria of *Spelotrema simile* (Jägerskiöld, 1900) Looss, 1902. (Described as *Microphallus similis* in Stunkard [1970]). At least 2 generations of sporocysts, contain yellow pigment. Cercaria, described as *Cercaria ubiquita* Lebour, 1907; oval to ovate; monostomatous; non-oculate; simple tail; integument spinous; 4 pairs of penetration glands; numerous cystogenous glands; excretory vesicle V-shaped; flame-cell formula 2 [(1 + 1) + (1 + 1)]; in water currents enter the gill chambers of green crabs, *Carcinides maenas,* penetrate and migrate to all parts of the body and encyst. Metacercaria in oval cysts, the digestive tract and acetabulum develop, excretory vesicle enlarges with lateral evaginations, flame-cell formula 2 [(2 + 2) + (2 + 2)]; natural hosts, *Larus argentatus* and *Sterna hirundo,* experimental in white mice. *(Stunkard, 1957b).

In *LITTORINA SAXATILIS:*

1. Cercaria of *Cryptocotyle lingua.*
2. Cercaria of *Spelotrema simile.*
3. *Cercaria parvicaudata.*
4. Cercaria of *Podocotyle atomon* (Rudolphi, 1802) Odhner, 1905. Repeated generations of sporocysts, oval to elongate, apical birth-pore with sphincter; cercariae oval; distomate; suckers about same size, stylet double pointed, acetabulum postequatorial; integument without spines, with papillae bearing setae; 3 pairs penetration glands; tail short, with glands; excretory vesicle oval, lined with large, glandular cells; collecting ducts mesostomate; flame-cell formula 2 [(2 + 2) + (2 + 2)]; penetrate and encyst in amphipods, *Gammarus* spp., *Carcinogammarus mucronatus, Amphithoe longimana;* metacercariae may become progenetic; natural infections in fishes; experimentally in eels and sticklebacks. *(Hunninen and Cable, 1943a).
5. Cercaria of *Odhneria odhneri* Travassos, 1921. Cercariae develop in oval daughter sporocysts, the largest 0.40 by 0.016 mm, with apical sphincter and birth-pore. Cercaria small, oval, with a very small tail, curved and pointed at the tip; monostomate; oral sucker with small curved stylet; no pharynx or digestive ceca;

2 pairs of penetration glands on each side; excretory vesicle V-shaped; flame-cell formula 2 [(1 + 1) + (1 + 1)]. The cercariae cannot swim, but move about on the floor of the bay and are eaten by small crustaceans, especially shrimp, *Palaemonetes vulgaris.* In the body cavity of the shrimp cercariae grow; they are encysted, the digestive system with prepharynx, pharynx, and ceca which terminate at the acetabular level; the gonads are well formed and the flame-cell formula is completed 2 [(2 + 2) + (2 + 2)]; adults were obtained experimentally in young gulls, occur naturally in shore-birds. *(Stunkard, 1979).

In *LYONSIA HYALINA:*

Sporocysts of *Rhipidocotyle transversale* Chandler, 1935 and *Rhipidocotyle lintoni* Hopkins, 1954 occur as tangled masses of colorless, branching tubules in the gonad and digestive gland of *L. hyalina.* The tips of the branches are centers of rapid cell-division and growth of the sporocyst proceeded as tubules invaded the haemocoele of the clam. The lumen of the branches is variable and contains both germ-balls and developing cercariae. The cercariae are cylindrical, flattened, with spinose integument; in a specimen 0.10 mm long and 0.030 mm wide, the tail-stem is bilobed and the furcae about as long as the body and curved medially in the characteristic crossed condition. In fully developed cercariae the body is elongate, 0.12–0.25 mm long and 0.03–0.07 mm wide; the tail-stem is 0.02–0.035 mm long; the furcae arise from the anterolateral faces and when extended may be ten times as long as the body. There is an anterior penetrantorium; the mouth is midventral; the intestine saccate; the reproductive organs represented by a curved mass of germinal cells in the posterior part of the body; the flame-cell pattern is 2 [(2 + 2) + (2 + 2)]; the furcae retract in spiral manner and the cercaria cannot swim. The furcae attach to the surface of a fish, typically *Menidia menidia,* and retract; the cercariae penetrate and form encysted metacercariae in the muscles; as development proceeds, the juveniles assume the features that distinguish the two species in the intestine of gars, *Strongylura marina.* (Stunkard, 1976).

In *MITRELLA LUNATA:*

1. Cercaria of *Opecoeloides vitellosus* (Linton, 1900) Von Wicklen, 1946. (Syn. *Cymbephallus vitellosus* [Linton, 1900], Linton, 1934. *Anisoporus manteri* Hunninen and Cable, 1940). Daughter sporocysts, oval to elongate in digestive gland of *M. lunata;* cercaria oval to ovate, distomate, cotylurous, tail shorter than body; non oculate; suckers approximately equal, oral sucker with double pointed stylet; integument without spines; 3 pairs of penetration glands; excretory vesicle oval to elongate, collecting ducts mesostomate, ciliated, flame-cell formula 2 [(2 + 2) + (2 + 2)]. Migrate to haemocoele, encyst and develop as metacercaria in amphipods, *Gammarus* spp., *Carcinogammarus mucronatus, Amphithoe longima;* metacercaria grow and develop digestive system, ceca fuse posteriorly with excretory vesicle to form a uroproct; adults in *Fundulus* spp., and other marine fishes. (Hunninen and Cable, 1941).

2. Cercaria of *Zoögonoides laevis* Linton, 1940. Sporocyst developed in haemocoele of *M. lunata* after ingestion of embryonated eggs, successive generations of daughters; cercaria distomate, tailless; non-oculate, oral sucker with simple stylet; acetabulum large, in posterior half of body, integument spinose; esophagus and ceca long; cystogenous cells distributed over body; openings on dorsal and ventral surfaces; 8 pairs of penetration glands, anterior and lateral to acetabulum; excretory vesicle saccate, wall with large epithelial cells, flame-cell formula 2 [(2 + 2) + (2

+ 2)]; metacercariae encysting in body-wall and parapodia of *Nereis virens;* adults experimentally in *Tautoga onitis.* *(Stunkard, 1943).

3. Cercaria of *Neopechona cablei* Stunkard, 1980. Daughter rediae simple, elongate, birth pore ventral, postpharyngeal; flame-cell formula 2 [(1 + 1) + (1 + 1)]. Cercaria distomate, biocellate, trichocercous, tail longer than body with 21 tufts of lateral setae and an immobile terminal tuft; undetermined number of penetration glands; no cystogenous glands; suckers subequal; pharynx short; ceca long; excretory vesicle saccate; collecting ducts mesostomate; flame-cell formula 2 [(4 + 4 + 4) + (4 + 4 + 4)]; cercariae emerge from redia while immature, before ocelli are recognizable, develop in haemocoele, emerge through gills of snail at night; swim in successive bursts, at all levels in the water, tail first, body contracted; tip of tail adheres to debris and to surface of medusae and ctenophores; larva penetrates and when body is firmly embedded the tail lashes free and swims away. Experimentally cercariae entered medusae of *Podocoryne carnea* and were later fed to scup, *Stenotomus chrysops,* where they became mature. *(Stunkard, 1980b).

In *MYA ARENARIA:*

1. *Cercaria myae* Uzmann, 1952. Distomate, furcocercous; integument spined; short, wide ceca; 2 pairs of penetration glands; excretory vesicle V-shaped with long arms; tubule in tail stem, pores at tips of furcae; develop in motile, unpigmented, clavate sporocysts in the digestive gland and gonads of *Mya arenaria;* further development and adult unknown. *(Uzmann, 1952).

In *MYTILUS EDULIS:*

1. Cercaria of *Proctoeces maculatus* (Looss, 1901) Odhner, 1911. (Syn. *Cercaria milfordensis* Uzmann, 1953). Repeated generations of simple, saccate motile, orange colored sporocysts; cercariae distomate, microcercous, smooth integument; 6 pairs of penetration glands; excretory vesicle Y-shaped with long arms; flame-cell formula 2 [(2 + 2) + (2 + 2)]; metacercariae in *M. edulis* and other mollusks, also annelids; may become gravid; progenetic. Adults in *Labrus merula* and other mollusk ingesting fish. *(Uzmann, 1953; Stunkard and Uzmann, 1959).

In *NASSARIUS OBSOLETUS:*

1. *Gynaecotyla adunca* (Linton, 1905) Yamaguti, 1939. (Syn. *Cercaria nassicola* Cable and Hunninen, 1938). Life-cycle by Rankin, (1940b); confused and erroneous account by Hunter and Vernberg, 1953. Successive generations of sporocysts in digestive gland and gonad of snails. Cercariae tiny, monostomate, acetabulum and digestive tract not yet developed; non-ocellate; tail simple; integument spinose; stylet relatively large, lancet-shaped with ventral keel; 4 pairs of penetration glands; enter branchial lamellae of amphipods; encyst in *Talorchestra longicornis;* excretory vesicle V-shaped, collecting ducts mesostomate, formula 2 [(1 + 1) + (1 + 1)]; develop to metacercaria; excretory formula 2 [(2 + 2) + (2 + 2)]; experimentally in young gulls; naturally in shore-birds. *(Rankin, 1940b).

2. *Himasthla quissetensis* (Miller and Northup, 1926) Stunkard, 1934. (Syn. *Cercaria quissetensis* Miller and Northup, 1926.) Daughter rediae, with locomotor appendages, migrate actively; as they mature the body-wall contains orange pigment and the birth-pore becomes functional; attain a length of 1.0–1.5 mm and a width of 0.1–0.25 mm. Cercariae emerge from snail through the gills, swim actively 6–12 hours; body elongate oval in outline, with small papillae on body and tail, each with

a delicate bristle; distomate with reniform collar bearing 31 peristomial spines; simple tail with excretory pores on sides; Many penetration and cystogenous glands; excretory vesicle small, oval with a short anterior stem, flame-cell pattern not determined. Metacercariae in various mollusks, adults in gulls. *(Stunkard, 1938a).

3. *Lepocreadium setiferoides* (Miller and Northup, 1926) Martin, 1938. (Syn. *Cercaria setiferoides* Miller and Northup, 1926.) Develop in daughter rediae, no collar or feet; cercariae distomate, suckers subequal; ceca rudimentary; gymnocephalous, oculate; with long setiferous tail; 8 pairs of penetration glands; scattered cystogenous glands; excretory vesicle tubular, contains concretion; flame-cell formula 2 [(3 + 3 + 3 + 3) + (3 + 3 + 3 + 3)]; cercariae penetrate and encyst in triclad turbellarians and spionid annelids; experimental infections in small flounders. *(Martin, 1938).

4. *Stephanostomum dentatum* (Linton, 1900) Linton, 1940. (Syn. *Cercaria dipterocerca* Miller and Northup, 1926.) Asexual generations in rediae; small specimens mobile, older rediae with yellow to orange pigment; cercariae biocellate; distomate; suckers subequal; no stylet; ceca not developed; integument spinose on body and tail; lophocercous; tail short with finfolds; circumoral area without spines, then two alternate rows, each with 27 spines; excretory vesicle V-shaped; tubules stenostomate, flame-cell formula 2 [(3 + 3 + 3) + (3 + 3 + 3)]; excretory pores on sides of tail; cercariae penetrate and encyst in *Menidia menidia;* adults in puffers, *Spheroides maculatus.* *(Stunkard, 1961).

5. *Stephanostomum tenue* (Linton, 1898) Linton, 1934. Daughter rediae oval to elongate; cercariae distomate, suckers subequal; tail simple, body with eye-spots and stylet; integument with papillae and short lateral bristles; oral sucker with two rows of alternating spines; excretory vesicle Y-shaped with cuboidal vesicular cells; metacercariae in *Fundulus* spp. and *Menidia menidia;* adults in *Spheroides maculatus.* *(Martin, 1939).

6. *Austrobilharzia variglandis* (Miller and Northup, 1926) Penner, 1953 (Syn. *Cercaria variglandis* Miller and Northup, 1926.) Asexual generations in sporocysts, simple, oval to elongate; cercariae distomate; oculate; apharyngeate; brevifurcate; tail same length as body; six pairs of penetration glands; excretory vesicle small with islet of Cort; flame cell formula 2 [(3 + 3) + (2) + (1)]; no metacercarial stage; adults dioecious; in mesenteric veins of canaries, pigeons, and gulls. *(Stunkard and Hinchliffe, 1952).

7. *Zoogonus rubellus* (Olsson, 1867–68) Odhner, 1902. (Syn. *Distomum lasium* Leidy, 1891.) Eggs have no shells; mature miracidia are in membranous sacs; penetrate *N. obsoletus,* where repeated generations of sporocysts produce cercariae, which leave the sporocysts early and mature in the haemocoele of the snail. When mature, they emerge through the gills and excurrent siphon; cercariae distomate; acetabulum slightly larger than oral sucker; ceca short; six pairs of penetration glands; cystogenous glands plentiful; posterior end of body cupuliform, serves for attachment; excretory vesicle saccate, collecting ducts mesostomate, flame-cell formula 2 [(2 + 2) + (2 + 2)]; metacercariae in body-wall and parapodia of *Nereis virens;* adults in eels and toadfish. *(Stunkard, 1938b).

8. *Diplostomum nassa* (Martin, 1945) Stunkard, 1973. Syn. *Cercaria nassa* Martin, 1945. The only marine strigeid cercariae described from the Atlantic coast of the United States; rare; examination of 10,000 snails over a three year span yielded only four infections; definitive hosts mostly absent; asexual generations in sporocysts; cercariae 0.06–0.026 by 0.03–0.05 mm; distomate; longifurcata; body and tail-stem about same length, furcae almost as long as tail stem; acetabulum postequatorial; integument spinous, 8 preacetabular and 4 postacetabular annular

rows of longer spines; 8 pairs of penetration glands; flame-cell formula 2 [(2 + 2) + (2 + 2) + (2)]. Cercariae attack small fishes, drop the tails, but no experimental metacercariae. Life-history unknown. *(Martin, 1945; Stunkard, 1973).

In *NASSARIUS TRIVITATTUS:*

1. *Lepocreadium areolatum* (Linton, 1900) Stunkard, 1969. For more than fifty years the snails of the Woods Hole area have been examined for infections by digenetic trematodes. Infections in *N. trivitattus* are rare; only two specimens, one in 1978 and one in 1979 were found shedding cercariae of *L. areolatum;* the cercariae are produced in elongate rediae; attenuate at both ends, with a curved taillike posterior tip; cercariae distomate; suckers subequal; acetabulum postequatorial, integument spined in anterior fourth of body; biocellate; trichocercous; tail longer than body with 27 pairs of lateral setae and a terminal tuft; four pairs of penetration glands; prepharynx and esophagus long; ceca open into the excretory bladder; excretory vesicle tubular, may extend to the acetabulum, collecting ducts mesostomate; flame-cell formula 2 [(5 + 5 + 5) + (5 + 5 + 5)]; cercariae penetrate ctenophores and medusae which serve as paratenic hosts; adults in fish, *Morone americana, Tautoglabrus adspesus,* and *Menticirrhus saxatilis.* *(Stunkard, 1980a).

2. *Tubulovesicula pinguis* (Linton, 1940) Manter, 1947. The life-history of this species has been under investigation since Sinclair *et al.* (1972) predicated that it is a stage in the development of *Stomachicola rubeus* (Linton, 1910) Manter, 1947. Since that time only one snail (from 10,000) has been taken that was shedding encysted hemiurid cercariae. Three other infected snails have been found, but the infections were immature, and no other cercariae have been taken. The cercariae are produced in successive generations of rediae. When mature they emerge from the rediae into the haemocoele of the snail but they do not accumulate. At night they emerge from the gills and almost immediately they encyst, so all swimming cercariae were already encysted. The encysted cercariae were fed to copepods, *Acartia tonsa,* where the larvae were expelled through a long delivery tube, enclosed in the cyst. The cercariae migrated to the haemocoele and developed, but were not yet infective when fed to fishes. Eggs from natural infections were embryonated and fed to laboratory raised *N. trivatattus;* small sporocysts were recovered. Adults occur in *Menidia menidia, Fundulus heteroclitus,* and other fishes. *(Stunkard, 1980c).

In *ODOSTOMIA TRIFIDA:*

1. *Lecithaster confusus* Odhner, 1905. Successive generations of rediae, cylindrical with tapering ends, unpigmented; cercariae cystophorous, cysts average 50 μ in diameter; fed to copepods, *Acartia tonsa;* eversion of delivery tube frees cercaria; migrates to haemocoele; does not encyst; grows to infective conditions; metacercaria spinose; suckers subequal; ceca long; Excretory vesicle Y-shaped, arms unite anteriorly; adults experimentally in sticklebacks, *Apeltes quadracus, Fundulus* spp. and other fishes. The first life-cycle in the family Hemiuridae. *(Hunninen and Cable, 1943b).

In *PECTEN IRRADIANS LAMARCK:*

1. *Cercaria martini* n. sp. A previously unnamed cercaria. One reported infection, found by Linton, 10 August 1910 in a large scallop from Quisset Harbor, Buzzards Bay; sporocyst oval to elongate, 0.30 × 0.16 to 0.70 × 0.42 mm, large, extended specimen under coverglass pressure 1.78 × 0.36 mm; contracted slowly,

tended to be arcuate; cercaria, body slender, tail brevifurcate, may be extended and 2–3 times length of body; fin-like membranes on body and furcae; Anterior end of body bent ventrally with "pecking movements"; cercariae resemble *C. cristata.* *(Linton, 1915b).

In *SOLEMYA VELUM* SAY:

1. *Cercaria solemyae* Martin, 1944. Small, oval sporocysts in digestive gland of clam; cercariae average size 0.094 × 0.025 mm, fixed and stained, 0.06 mm long; tail very small, vestigeal; body integument spined; lateral edges crenate, set with short rows of longer spines; no performatorium; esophagus long; ceca short, bulbous; five pairs of penetration glands; excretory vesicle bilobed, tubule in tail divides to form two lobular sacs, no excretory pores. *(Martin, 1944b).

In *THAIS LAPILLUS* LINNAEUS:

1. Cercaria of *Renicola thaidus* Stunkard, 1964; successive generations of elongate sporocysts; cercaria oval, distomate, with stylet; suckers subequal; integument spined; tail simple, about body length; undetermined number of penetration glands, preacetabular; cystogenous glands numberous in postacetabular region; excretory vesicle Y-shaped; flame-cell formula 2 [(3 + 3 + 3) + (3 + 3 + 3)]; metacercariae encysted in *Mytilus edulis* and *Pecten irradians.* Adults experimentally in kidneys of gulls. (Stunkard, 1964b).

In *UROSALPINX CINEREA:*

1. *Parorchis avitus* Linton, 1914. (Listed as *Parorchis acanthus* [Nicoll, 1906] Nicoll, 1907 in Stunkard 1970). (syn. *Cercaria sensifera* Stunkard and Shaw, 1931.) Linton (1914) reported that the miracidium while still in the egg, in the uterus of the worm, contains a single, well developed redia. There is no sporocyst; generations of daughter rediae are produced; cercaria with peristomial collar, integument spined; distomate; no ocelli or stylet, esophagus long; ceca long; excretory vesicle saccate; collecting ducts mesostomate, with concretions; flame-cell formula 2 [(3 + 3 + 3) + (3 + 3 + 3)]; cercariae encyst on solid objects; adults in terns, *Sterna hirundo,* and gulls *Larus argentatus.* *(Stunkard and Shaw, 1931; Stunkard and Cable, 1932).

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POSTFERTILIZATION CHANGES IN THE HORSESHOE CRAB *LIMULUS POLYPHEMUS* L.

GEORGE G. BROWN[1,2] AND SUSAN RUTTENBERG BARNUM[1]

[1]*Department of Zoology, Iowa State University, Ames, Iowa 50011, and*
[2]*Hopkins Marine Station, Stanford University, Pacific Grove, California 93950*

ABSTRACT

Postfertilization changes occurring during early development in the American horseshoe crab, *Limulus polyphemus*, have not been completely clarified in the literature. To help alleviate this problem, the present study examines the first four of twenty-one developmental stages as outlined for the Japanese horseshoe crab, *Tachypleus tridentatus*, by Sekiguchi (1973) and for *Limulus* by Brown and Clapper (1981). These four stages involve cleavage (Stages 1–3) and early blastulation (Stage 4). Cleavage consists of two parts: 1) intralecithal cleavage and 2) total cleavage. The first part (Stages 1 and 2) involves nine postfertilization events which are emphasized in this study and include the cortical reaction and three intermittant granulations. During the first two granulations, granules appear simultaneously over the entire surface of the fertilized egg. The third granulation differs from these in that granules appear locally on the embryo and gradually progress over the surface. This granulation is also characterized by a contraction wave passing over the surface. The first part of cleavage (intralecithal cleavage) ends with the appearance of nuclei near the surface (Stage 2). The second part of cleavage (total cleavage, Stage 3) then proceeds but is somewhat modified from a standard holoblastic cleavage since the planes of each division are not completed.

INTRODUCTION

In a recent review on chelicerate embryology, Anderson (1980) states that "in spite of the excellent recent work of Scholl (1977), the critical early stages of xiphosuran development are still not clear." In the present study, the sequence of dynamic postfertilization changes occurring to the *Limulus polyphemus* embryonic surface during early development has been examined. These changes have been related to cleavage, a process in *Limulus* and other xiphosurids (*Tachypleus tridentatus* and *T. gigas*) which definitely needs clarification. For this study, the authors have supported the following two-part classification of xiphosurid cleavage, as modified from a review by Anderson (1973): 1) "intralecithal cleavage" which includes all postfertilization changes occurring in Stages 1 and 2 (Sekiguchi, 1973; Brown and Clapper, 1980); and, 2) "total cleavage" which includes Stage 3 and terminates with the appearance of Stage 4 or early blastula formation. Although this present approach provides insight on the overall cleavage process with emphasis on surface changes, further investigations are necessary to link these changes with nuclear divisions and migrations.

In reference to early development of xiphosurids, earlier investigators (Brooks and Banks, 1885; Osborn, 1885; Kishinouye, 1891; Kingleys, 1892; Iwanoff, 1933) reported a series of "furrowings" which they interpreted as cleavage and which agrees

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with total cleavage as presented in this study. However, among these investigators only Kingsley (1892) described any postfertilization changes preceding total cleavage. In particular, he described a columnar layer covering the embryonic surface (granulation in the present study) that appeared and disappeared within a few hours after fertilization. He also briefly described the presence of nuclei during early development and their migration to the embryonic surface (intralecithal cleavage). Unfortunately, possibly due to the difficulty of preparing materials, he did not pursue further studies on these postfertilization events. More recently, various aspects of intralecithal cleavage have been examined (Sekuguchi, 1960; 1973; Brown and Clapper, 1980; 1981; Sekiguchi *et al.,* 1982), but the emphasis has not been on the granulation processes as outlined in the present study.

Materials and Methods

Source of animals

Adult specimens of *Limulus polyphemus* L. were shipped from the Florida Marine Biological Specimen Co., Inc., Panama City, FL, during the summer and fall months and were kept at 15°C in 150 gal Instant Ocean Aquaria containing artificial sea water (ASW) from Jungle Laboratories Corporation, Comfort, TX. The animals were maintained following the procedures previously outlined by Brown and Clapper (1981).

Gamete collection, fertilization, and early development

Gametes were collected by brief electrical stimulation (3–4 V, 0.2–1.0 mA, ac) in a region proximal to the genital pores. Semen was diluted with ASW to a 10% sperm suspension (10^9 spermatozoa/ml) and was generally used within 5 min. With wooden applicators, approximately 10–25 freshly spawned eggs were collected and placed in a plastic Petri dish containing ASW. The dish was swirled to disperse oviductal fluid, one or two drops of a sperm suspension were added, and the dish was swirled again to assure mixture of gametes. The ASW was subsequently aspirated and fresh ASW was added. To enhance the observation of surface features and nuclei, some embryos were stained (Sekiguchi, 1960) for about 2 h shortly after fertilization using one of the following vital stains: Nile blue A or neutral red (.025 mg/ml of ASW). Embryos were examined with a Wild M-5 dissecting microscope and at selected times after fertilization: 0, 15, 25, 90 min and 3, 4, 6, 12, 14.5, 20, 28, 48 h, were photographed using a Nikon camera. For all observation of develomental stages the temperature was maintained at 22–23°C.

Histology

Aliquots of fertilized eggs or embryos at the following times after fertilization: 12, 40 min and 1.5, 3, 4, 6, 9, 15, 18, 27, 48 h were removed and fixed in ice cold 2.5% glutaraldehyde buffered with 0.2 *M* sodium cacodylate (pH 7.2). Unfertilized eggs were also treated similarly. To facilitate the penetration of fixative, egg envelopes were removed from the embryos with watchmaker forceps 10–40 min after initiating the fixation procedure. After 40 min of fixation, embryos were often brittle and as a result were frequently damaged during removal of the egg envelopes. In unfertilized and recently fertilized eggs (12 min), egg envelopes adhered so tightly to the surface, they were often partially removed in order to prevent extensive damage to the surface. After removal (or partial removal) of egg envelopes, specimens were placed in fresh fixative and stored overnight at room temperature prior to clearing with ethyl alcohol and embedding in Polysciences JB-4 plastic embedding water-soluble medium. Embryos were serial sectioned using a Sorvall M-1, stained with 1% toluidine blue with 1% borax, and subsequently examined and photographed with a Nikon camera mounted on a Zeiss WL compound microscope. The embryos were carefully monitored at each step of preparation to ensure that the above procedures were not altering the appearance of the embryonic surface.

Time-lapse cinematography

The timing of the initial appearance of postfertilization events and embryonic stages and their duration were augmented by using time-lapse cinematography. Developing embryos were photographed with an Arri-S 16 mm camera equipped with an Arri single-frame motor which exposed one frame at a time upon receiving an electrical pulse from an intervalometer. A disk intervalometer was used for filming at rates of one frame every 5 s for the first 3 h of *Limulus* development and one frame every 15 s thereafter. In addition, a clock intervalometer was used for filming at the rate of one frame per min. Shutter speed was ¼ s. The Petri dish containing embryos was placed on a sheet of black plastic, the ASW was layered with oil to prevent evaporation, and the dish was illuminated at 20 degrees with an incandescent ribbon filament microscope lamp.

RESULTS

Stage 1, postfertilization events during intralecithal cleavage

Nine surface changes or phenomena represent this stage and are designated as "events." Although the first four events have previously been described using ultrastructural methods (Bannon and Brown, 1980; Brown and Clapper, 1980), their inclusion in this study is necessary to present a precise sequential development of *Limulus*.

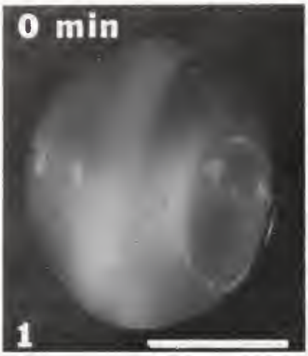
Event 1, unfertilized egg. When examining with a dissecting microscope, the egg surface (beneath the egg envelope) has a smooth appearance (Fig. 1). In histological sections, this surface is even with no bumps or depressions (Fig. 2). The thin distinct periplasm is approximately 5–10 μm thick and is similar in appearance throughout the egg periphery (Fig. 2).

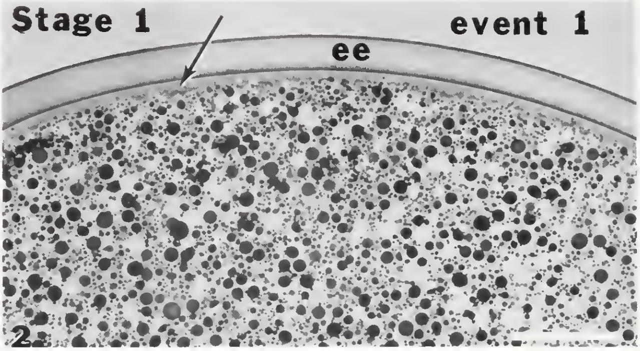
Event 2, appearance of pits. Approximately 10–15 min after fertilization, small pits are distributed over the entire surface of the fertilized egg (Fig. 3) and are slowly increasing in diameter. In sections (Fig. 4), these pits are hemispherical depressions measuring approximately 80–100 μm in diameter and 30–40 μm in depth. These depressions have caused the periplasm and the underlying yolky layer to become very uneven. Besides this unevenness, the periplasm is also quite variable in thickness (5–25 μm).

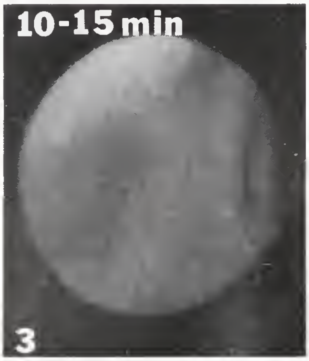
Event 3, coalescence of pits. The pits continue to enlarge and coalesce with neighboring pits forming large crater-like surface areas (Fig. 5). Although the embryonic surface is still very uneven (Fig. 6), the periplasm has now become less variable in thickness (5–15 μm) when compared to event 2.

Event 4, smooth surface after the cortical reaction. By approximately 45–60 min after fertilization, the embryonic surface appears smooth and even (Figs. 7, 8). The periplasm has continued to decrease in thickness and is now once again (as in event 1) a thin peripheral layer (5–10 μm thick) around the embryo (Fig. 8).

Event 5, first granulation. The first granulation process appears about 2.5–3.0


0 min
1


Stage 1
event 1
ee
2


10-15 min
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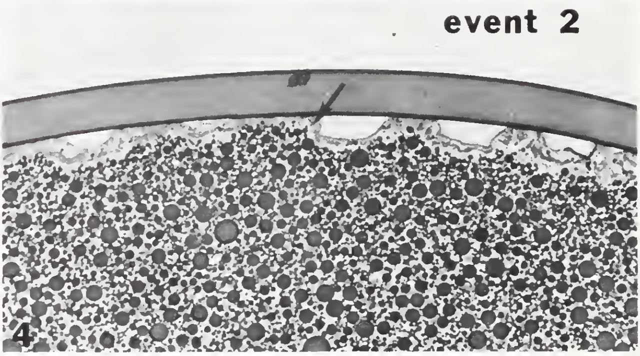
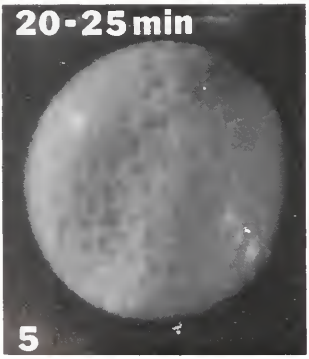
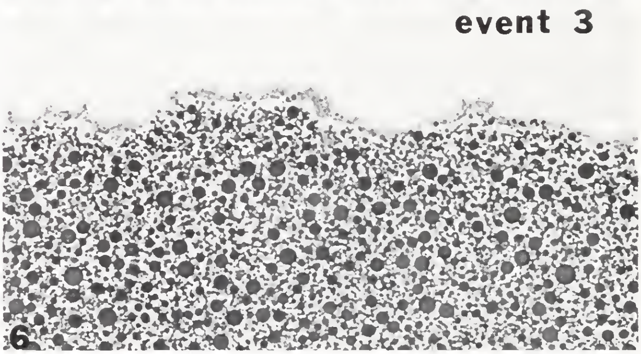
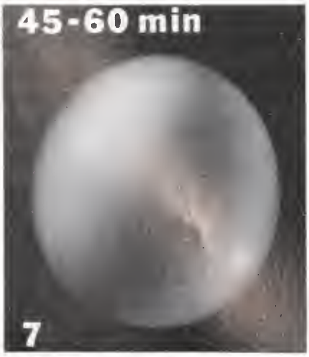
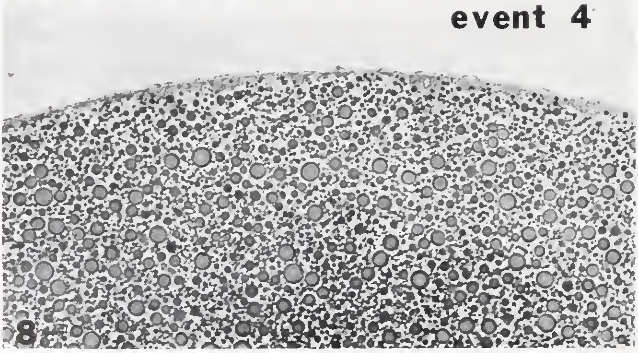

event 2
4


20-25 min
5


event 3
6


45-60 min
7


event 4
8

h after fertilization (Figs. 9, 10). Granules appear simultaneously over the entire embryonic surface as small nodules and increase in size eventually (10–15 min) resulting in the formation of a columnar layer (Fig. 10) which gives a corregated appearance to the periphery of the embryo (Fig. 9). Although these granules are somewhat variable in size and shape, most measure approximately 60–75 μm in length and 25–35 μm in diameter. Their contents (yolk platelets and other cytoplasmic components) are continuous with the underlying yolky material, indicating that the granules at this time are not separate membrane-bounded structures (Fig. 10). The periplasm has become greatly reduced (<5 μm thick). After being visible for approximately 1.0 h, all the granules simultaneously recede and disappear.

Event 6, smooth surface after first granulation. At 3.5–4.0 h after fertilization the embryonic surface for the second time since the cortical reaction appears smooth (Figs. 11, 12). Although similar to event 4, numerous cytoplasmic cylinders (5–10 μm wide) which are free of yolk platelets and extend approximately 30–35 μm into the cytoplasm (Fig. 12) are present and are associated with the periplasm (Fig. 12). The periplasm is much more distinct than in the previous event and forms a thin peripheral layer. This event lasts approximately 1.5 h.

Event 7, second granulation. The second granulation is initiated 5.0–5.5 h after fertilization. As in event 5, small granules appear simultaneously over the entire embryonic surface, slowly increase in size, and 10–15 min after their initial appearance form a columnar layer (Figs. 13, 14) very similar to the one in event 5 except the granules are somewhat larger and more uniform in size (approximately 75–100 μm in length and 25–40 μm in diameter). The clear cytoplasmic cylinders

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FIGURES 1–8. Stage 1, postfertilization changes during intralecithal cleavage. The approximate time after fertilization when an event is initiated is placed in the upper left hand corner of odd-numbered micrographs.

Event 1, unfertilized egg:

FIGURE 1. A typical appearance of a recently spawned egg. The large indentation on the right side is due to the packed condition of eggs in the oviduct. This indentation usually disappears in the first 30–60 min. The encompassing egg envelope is transparent and can be observed near the indentation. Beneath this envelope, the surface of the egg has a smooth appearance. Bar = 1.0 mm.

FIGURE 2. In this egg section, three distinct layers or structures are observed: the outer egg envelope (ee), the periplasm (arrow), and the inner yolky mass representing the bulk of the egg. The periplasm is distinguished by the lack of yolk platelets. Note: a peculiarity in the fixation of unfertilized eggs renders large numbers of yolk platelets transparent whereas in micrographs of fertilized eggs, these yolk platelets appear dense. Bar = 0.1 mm.

Event 2, initiation of cortical reaction and appearance of pits:

FIGURE 3. Once the egg is fertilized, the cortical reaction occurs resulting in the simultaneous appearance of numerous pits over the entire surface of the fertilized eggs. In this micrograph the pits appear as individual dark blotches.

FIGURE 4. Beneath the egg envelope in this sectioned fertilized egg, depressions (pits) have formed which have forced both the periplasm (arrow) and the surface of the yolky layer immediately below the periplasm to become uneven.

Event 3, coalescence of pits:

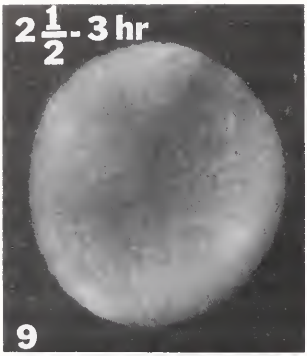
FIGURE 5. The blotchy areas representing pits have now increased in size and are variously shaped, a condition due to pits growing into one another (coalescing) and forming craters.

FIGURE 6. The surface of the fertilized egg is still uneven. In this and all following sectioned material, the egg envelope has been removed, a process which did not cause any noticeable changes in the features of the embryonic surface features.

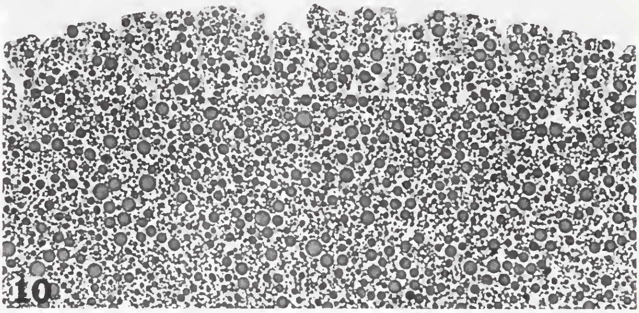
Event 4, completion of the cortical reaction:

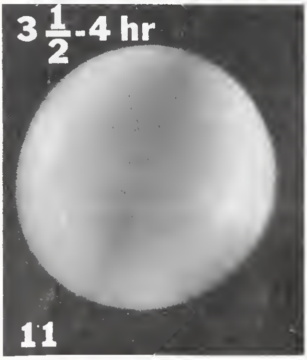
FIGURE 7. The fertilized egg surface is no longer distinguished by the presence of pits or craters and is characterized by a smooth appearance.

FIGURE 8. The periplasm and underlying yolky layer no longer form an uneven surface, but have an appearance similar to the sectioned unfertilized egg.

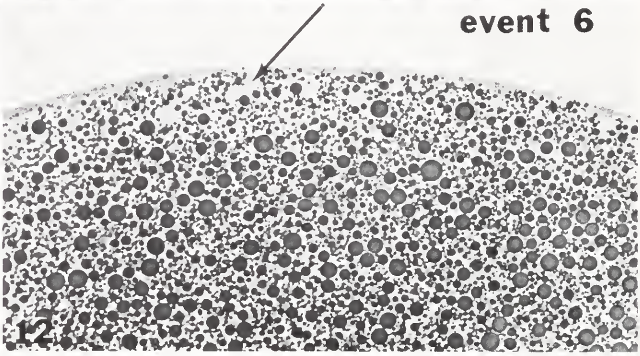


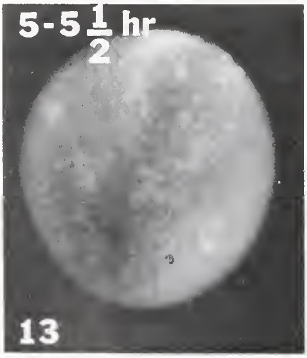
Stage 1 event 5



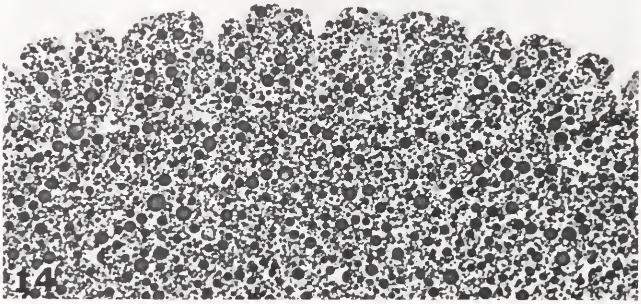


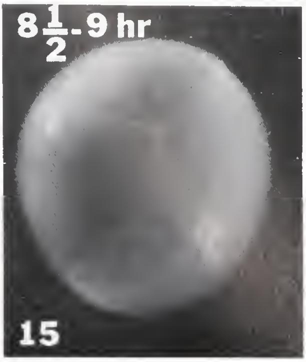
event 6



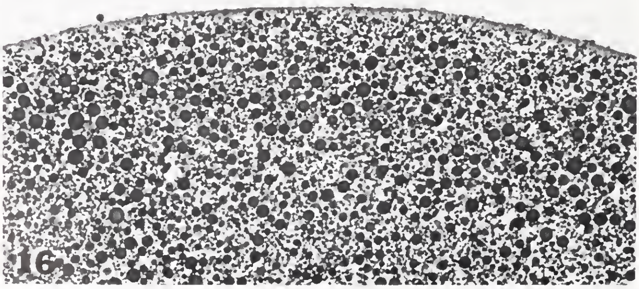


event 7





event 8



observed in event 6 have greatly reduced in thickness (Fig. 14). This second granulation lasts for approximately 3 h.

Event 8, smooth surface after the second granulation. At 8.5–9.0 h after fertilization, the embryonic surface for the third time since the cortical reaction has a smooth appearance (Figs. 15, 16) and very closely resembles events 4 and 6 except that the cytoplasmic cylinders (presence in event 6) are absent. The periplasm has increased to its original thickness (5–10 μm) around the embryo (Fig. 16).

Although serial sections of previous events were very carefully examined, intralecithal nuclei were observed for the first time in this event. Although not demonstrated with a micrograph, these oval-shaped nuclei (8 × 10 μm) are located within yolk platelet-free cytoplasmic masses (25–30 μm in diameter) and can be easily distinguished. In a serial sectioned embryo representing this event, 15 such nuclei were counted. Each nucleus was located approximately 300 μm (one sixth of embryonic diameter) from the surface.

Event 9, third granulation and contraction wave. Beginning 14–15 h after fertilization (Figs. 17, 18), the dynamic third granulation appears. In differing from the first two granulations, this one initiates in one locale on the embryo and slowly (approximately 1 h) spreads across the entire embryonic surface. In sections passing through the spreading edge of this granulation (Fig. 18), the individual granules are small and vary greatly in shape. As granules increase in size, a columnar layer similar to the two previous granulations forms, but is smaller in height (25–30 μm). Although the appearance of granules in this third granulation is readily observed, their disappearance has not yet been determined. Apparently, these granules persist throughout event 9 and into Stage 2 and possibly Stage 3.

When the granules blanket approximately one-third of the embryonic surface, a contraction wave becomes apparent near the origin of the granulation and precedes in the same direction as the granulation spreading edge. The contraction wave takes approximately 1 h to move over the embryonic surface and generally is prominent.

FIGURES 9–16. Stage 1: Events 5–8.

Event 5, first granulation:

FIGURE 9. Granules appear simultaneously over the entire embryonic surface giving the embryo a corrugated appearance.

FIGURE 10. Fifteen min after their initial appearance, the granules are shaped like cylinders and are continuous at their basal ends with the underlying yolky layer. As the granules form they enclose numerous yolk platelets and occupy the entire embryonic periphery.

Event 6, smooth surface after first granulation:

FIGURE 11. As the granules recede and disappear, the embryonic surface becomes smooth for the second time since the cortical reaction.

FIGURE 12. A smooth surface and irregularly-shaped cytoplasmic areas (arrow) which are extensions of the periplasm into the yolky layer are characteristics of this event.

Event 7, second granulation:

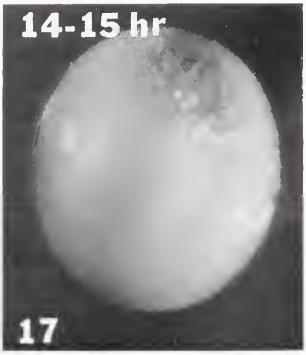
FIGURE 13. The second granulation also appears simultaneously over the surface and appears initially to be very similar morphologically to the first granulation (event 5). A major difference is the longer duration of the second granulation.

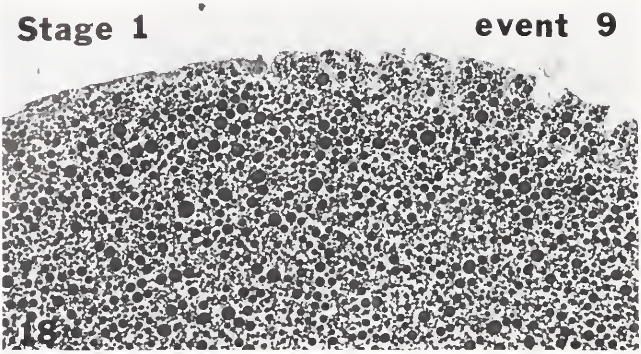
FIGURE 14. This sectioned embryo (as was the embryo for the first granulation) was fixed and prepared approximately 15 min after the initial appearance of granules. The granules for both events at this time of fixation are very similar.

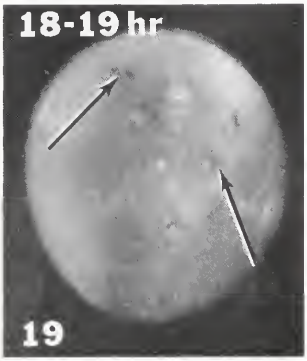
Event 8, smooth surface after second granulation:

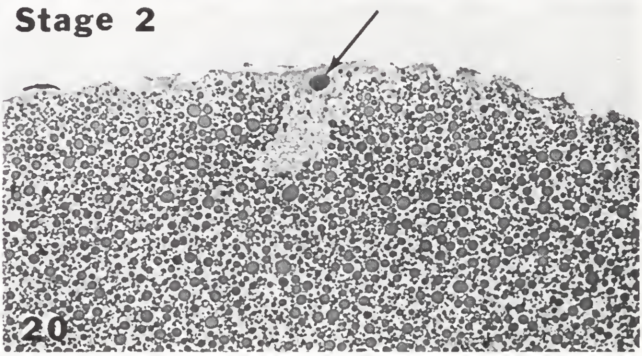
FIGURE 15. A smooth embryonic surface appears for the third time since the cortical reaction. An occasional "oil droplet" may be observed on the surface.

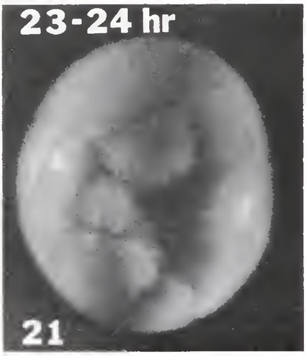
FIGURE 16. The thickness of the periplasm and the even surface give this sectioned embryo a resemblance to events 4 and 6, however, in the latter case, the irregularly-shaped cytoplasmic areas (Fig. 12) are absent.

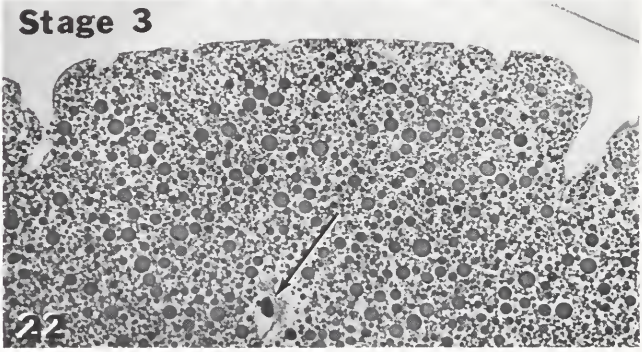

14-15 hr
17

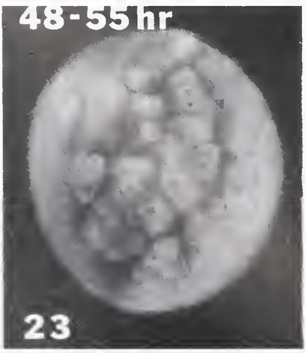

Stage 1
event 9
18

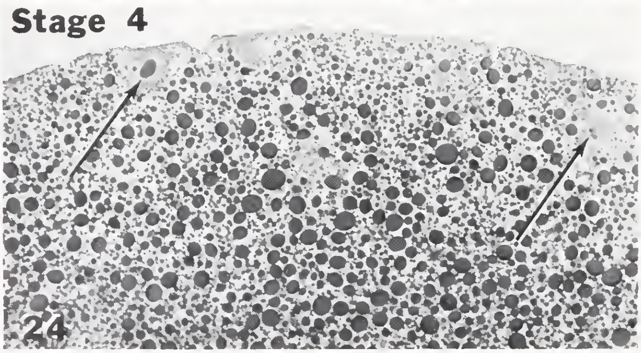

18-19 hr
19


Stage 2
20


23-24 hr
21


Stage 3
22


48-55 hr
23


Stage 4
24

The occurrence and movement of this contraction wave was overlooked in our studies and was not noticed until we viewed film produced by time-lapse cinematography.

As in event 8, intralecithal nuclei were identified. In one serial sectioned embryo, 13 such nuclei were counted. These were also located approximately 300 μm from the surface.

Stage 2, appearance of intralecithal nuclei on surface

About 18–19 h after fertilization, 12–16 intralecithal nuclei appear near the surface (Figs. 19, 20). In a sectioned embryo (Fig. 20), the cylindrical nucleo-cytoplasmic masses are in contact with the surface and extend approximately 52–62 μm into the yolky layer of the embryo. In one serial sectioned embryo, 14 intralecithal nuclei were counted. Thirteen of these were observed in close proximity to the surface and one nucleus, probably a "cleavage energid" (Anderson, 1973), was located near the center of the embryo. What may be confused with surface intralecithal nuclei are unidentifiable cell-like structures on the surface which resemble oil droplets with dark inclusions (Fig. 19).

Stage 3, "total cleavage"

Total cleavage commences when an irregular groove or furrow appears across the surface of the embryo. Upon initial examination, holoblastic cleavage appears to be occurring, however, observation of a sectioned 16-celled embryo shows that cleavage patterns are superficial (Fig. 22). Nevertheless, each blastomere of this embryo is associated with a cleavage nucleus. Also serial sections of 4 and 8 blastomere embryos (not demonstrated in this study) reveal 4 and 8 cleavage nuclei respectively associated with the blastomeres. These nuclei are believed to be daughter nuclei of the centrally located nucleus or cleavage energid described in event 9.

FIGURES 17–24. Stages 1 (Event 9), 2, 3, and 4.

Event 9, third granulation:

FIGURE 17. This granulation differs from the two previous granulations in that the origin is at one end of the embryo and slowly progresses over the entire surface. A contraction wave (not present) occurs in the same region and also passes over the embryonic surface.

FIGURE 18. The portion of the embryonic surface which demonstrates the leading edge of the granulation process.

Stage 2, appearance of intralecithal nuclei near surface:

FIGURE 19. Intralecithal nuclei (arrows) are near the embryonic surface. These nuclei are stained with Nile Blue A and appear as dark spots (arrows). As in this micrograph they are frequently paired. The upper left arrow designates one pair of nuclei while the lower right arrow demonstrates paired nuclei which are more separated than the first. The cell-like "oil droplets" observed on the surface are believed to be cytoplasmic blebs although they resemble small nucleated cells.

FIGURE 20. An intralecithal nucleus (arrow) is observed near the surface of this embryo. Surrounding the nucleus is a large amount of vesicular cytoplasm which extends into the yolky layer.

Stage 3, total cleavage:

FIGURE 21. Approximately 16 blastomeres are present in this embryo. Although total cleavage is initiated at 23–24 h after fertilization, this particular embryo has developed for 6–7 h beyond this time.

FIGURE 22. A cross section through one blastomere demonstrates that the cleavage plane is not complete. Note that the cleavage nucleus (arrow) is centralized in the blastomere block. Other nuclei which are believed to be descendants of the surface intralecithal nuclei are sometimes observed between blastomeres (not demonstrated in this micrograph).

Stage 4, early blastulation:

FIGURE 23. Nuclei in the blastomeres become obvious at the beginning of this stage. Although most blastomeres are similar in shape and size, patches of small blastomere are frequently observed.

FIGURE 24. Although the plasma membranes are difficult to observe in this section, individual blastomeres are represented by the nuclei (arrows).

Stage 4, early blastulation

Near the end of total cleavage, about 45–48 h after fertilization, when approximately 128 blastomeres are present (Fig. 23), the embryo is approaching the early blastula (Stage 4). The surface of each blastomere is so granular that internal features cannot be noticed when examining an embryo with the dissecting microscope. However, if one is patient and carefully observes this embryo, Stage 4 will appear as these granules slowly recede and the prominent nucleus (within a cytoplasmic mass) becomes obvious in each blastomere. These blastomeres vary in size, and areas of very small blastomeres can frequently be observed. In sections (Fig. 24), the blastomeres are observed only in the embryonic periphery.

DISCUSSION

The postfertilization events during early development in *Limulus* have revealed some significant aspects previously unknown or scantily studied. The cortical reaction in fertilized eggs of horseshoe crabs was invariably overlooked by earlier investigators and only recently has been generally described (Bannon and Brown, 1980; Brown and Clapper, 1980). An interesting aspect in this study is the overall view of the cortical reaction as revealed in sectioned embryos. This reaction is a dynamic process which affects a significant portion of the embryonic peripheral layer. For example, during the reaction the periplasm and the underlying yolky layer become very uneven. As demonstrated in an earlier study (Bannon and Brown, 1980), vesicles within the periplasm fuse together and by exocytosis empty their contents into the perivitelline region causing the formation and growth of pits. However, the very large pits must involve more material in their formation than what is originally present in the periplasm. Presumably, during the cortical reaction materials are continuously passing from the yolky layer into the periplasm by means of vesicles and then are secreted to the outside. This could account for the great displacement of the yolky layer observed.

As with the cortical reaction, the three granulations (Stage 1: events 5, 7, and 9) also represent dynamic changes involving a significant portion of the embryonic periphery. The first two granulations are similar during early initiation since granules appear spontaneously and are comparable in size and shape. The second granulation lasts longer and becomes more complex (Barnum, Clapper, and Brown, in preparation). Both granulations may involve some form of plasma membrane reorganization or possible secretory process. The latter suggestion is supported since protein synthesis increases during this period (Bannon, 1981; Bannon *et al.*, 1981), and may involve the secretion of precursors for proteins of the future extra-embryonic shell.

The third granulation (Stage 1, event 9) is unique in comparison with the previous two since the first appearance of granules is localized, and the movement of a contraction wave can be demonstrated. Contraction waves during cleavage or blastulation have been described in the developing embryos of a wide variety of animals. In barnacles, for example, a set of contraction waves occur shortly after fertilization, and their disruption with chemical inhibitors causes abnormal cleavage (Lewis, 1977). In *Xenopus* (Hara *et al.*, 1980), a "surface contraction wave" on the fertilized egg precedes the first cleavage division and occurs at the same time regardless of whether egg fragments, activated unfertilized eggs, or enucleated eggs are employed, implying the presence of a biological clock mechanism in the cytoplasm near the surface. Periodic morphological changes such as contractions or wave motions in the cytoplasm of fertilized eggs have also been measured in sea urchins (Yoneda *et al.*, 1978), in ascidians (Bell, 1962) and in insects (Fullilove *et al.*, 1978; Miyamoto and van der Meer, 1982). The observation of contraction waves has stimulated considerable interest in the mechanisms of cleavage and in the control of determination in embryos of several species. Preliminary studies of this phenomenon in *Limulus* indicate that the origin of the contraction wave may be related to the determination of the embryonic axis.

The appearance of intralecithal nuclei near the surface in *Limulus* marks the end of the postfertilization events (Stage 1) and the last step in intralecithal cleavage (Stage 2). This appearance of intralecithal nuclei has also been demonstrated in embryos of *Tachypleus* by Sekiguchi (1973). Unfortunately, in both species the fate of these intralecithal nuclei is unknown, although it must be different from the nuclei involved in total cleavage since some intralecithal nuclei remain near the surface and are observed between blastomeres during total cleavage (Stage 3). Further support for this hypothesis is provided in the examination of serial sectioned embryos during late intralecithal cleavage (Stage 2) and of embryos during total cleavage (Stage 3). In one Stage 2-embryo, 13 intralecithal nuclei were observed near the surface, and one nucleus (cleavage energid) was centrally located. In serial sectioned Stage-3 embryos (not demonstrated in this paper) undergoing 4 and 8 blastomere formation respectively, 4 and 8 cleavage nuclei were observed, each midway between the center and the outer edge of the embryo and appropriately placed in each blastomere quadrant or octant. These observations suggest that the original zygotic nucleus undergoes four intralecithal nuclear divisions, and at Stage-2 all nuclei migrate to the surface except one which becomes the cleavage energid for "total cleavage." Unfortunately, the tracking of intralecithal nuclei from the zygotic stage through the cleavage process by using the traditional Feulgen reaction has been unsuccessful due to nonspecificity of this reaction on our prepared materials. Also, the surface intralecithal nuclei observed in Stage 2 appear to lose their extensive cytoplasm mass early in Stage 3, and are difficult to demonstrate thereafter. The nucleus story in the xiphosurid is far from completion and certainly needs to be clarified. We are presently pursuing this problem using more sophisticated techniques.

The cleavage phenomenon in *Limulus* embryos is very unusual since both intralecithal cleavage and total cleavage occur during early development. As this seems to be uncommon in the Animal Kingdom, a comparison of *Limulus* cleavage to other species in this study will be limited to the arachnids. Since *Limulus* has a close phyletic relationship with the arachnids, it is interesting to note that both forms of cleavages are present in this group. However, they are only found independently. For example, total cleavage is rare and limited to specialized conditions such as very small yolk-free egg (<0.5 mm) of viviparous scorpions (*cf.* Anderson, 1973). In species of arachnids having larger eggs (0.5 mm–1.9 mm), the common pattern is intralecithal cleavage ending in the formation of a blastoderm. Studies by Holm (1952) on the agelenid spider, *Agelena labyrinthica*, and by Yoshikura (1954, 1955) on the liphistiid spider, *Hepathathela kimurai*, demonstrate that after the first three nuclear divisions, the resulting eight intralecithal nuclei are distributed equally in the fertilized egg. Interestingly, this is followed by the formation of several yolk pyramids which apparently have little, if any, relationship to the nuclei. In time, the pyramids break down as the nuclei migrate to the periphery and a cuboidal blastoderm forms around the embryonic periphery. No total cleavage occurs, although as suggested by Anderson (1973), the formation of yolk pyramids is reminiscent of this process. Perhaps even more interesting is the formation of numerous polygonal fields in the periplasm of the otenid spider, *Cupiennius salei* (Seitz, 1966), occurring a few hours after spawning during early intralecithal cleavage. The formation of these fields certainly is similar to the granulation process in *Limulus*. In summary, intralecithal cleavage and total cleavage in *Limulus* show similarities to nuclear division, pyramid formation, and polygonal fields in arachnids. More investigation is needed, however, before a clear comparison of cleavage between the two groups can be made.

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NEURAL CONTROL OF METAMORPHOSIS IN *DENDRASTER EXCENTRICUS*

ROBERT D. BURKE

Department of Biology, University of Victoria, Victoria, B. C., Canada V8W 2Y2, and
Bamfield Marine Station, Bamfield, B. C., Canada V0R 1B0

ABSTRACT

Glyoxilic acid induced fluorescence histochemistry and ultrastructural observations indicate that the larval nervous system includes an apical region of neuropile on the oral hood between the anterolateral arms and a ganglion comprised of nerve cell bodies and neuropile on the lower lip of the larval mouth. Electrical stimulation induced metamorphosis more frequently and with a lower mean threshold when a suction electrode was attached to either of these two nerve centers. When the entire oral hood, including oral ganglion and apical neuropile was excised, it and the posterior regions of the larva metamorphose spontaneously. Neither the excised pre-oral hood nor the remaining portions containing the oral ganglion and the adult rudiment metamorphose until exposed to the chemical cue that naturally induces metamorphosis. Excised larval arms do not respond to the natural cue. 10^{-5} M dopamine induced metamorphosis in a small proportion of larvae, and 10^{-5} M L-dopa and dopamine induced metamorphic responses in excised larval arms. It is proposed that the apical neuropile and oral ganglion are nerve centers that mediate between the perception of the natural cue and control the initiation of metamorphosis.

INTRODUCTION

The larvae of numerous species of marine invertebrates settle and metamorphose in response to certain factors associated with preferred adult habitats (Meadows and Campbell, 1972; Crisp, 1974; Chia and Rice, 1978). The paradigm is that larvae grow and develop to a state in which they are competent to metamorphose, and remain so until they encounter stimuli that induce metamorphosis (Crisp, 1974, 1976; Chia, 1978). Metamorphosis in most marine invertebrates is a relatively rapid sequence of developmental events that alters the form and function of the organism (Scheltema, 1974; Cloney, 1978). It has been surmised that these developmental events are held in abeyance by some endogenous physiological mechanism, though the nature of this control remains to be determined. It has been suggested that an endocrine system controls metamorphosis in decapod crustaceans in a manner similar to that of insects (Passano, 1961) but the evidence remains equivocal (Little, 1969). In other groups, neural control has been suggested (Hadfield, 1978; Morse *et al.*, 1979; Marsden and Anderson, 1981; Cloney, 1982).

Larval development and metamorphosis of several species of echinoids is typical of this model in many ways. Larvae undergo a predictable sequence of developmental changes during which a rudiment of the adult form develops on one side of the larva (MacBride, 1903, 1914, 1918). Competent larvae of several species metamorphose in response to cues from the adult environment (Caldwell, 1972;

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Cameron and Hinegardner, 1974; Highsmith, 1982). Metamorphosis is a rapid sequence of irreversible developmental events in which the adult rudiment is everted and the larval body retracted and resorbed (Cameron and Hinegardner, 1978; Chia and Burke, 1978; Burke, 1982). This report presents evidence indicating that the larval nervous system of the pacific sand dollar, *Dendraster excentricus* controls the initiation of metamorphosis.

MATERIALS AND METHODS

Larval culture

Larvae were reared from fertilized eggs using the standard procedures outlined by Strathmann (1968) and Hinegardner (1969). Adults were collected subtidally from Breaker Beach, Bamfield, British Columbia, Canada. Spawning was induced by intracoelomic injection of 0.55 *M* KCl. Larvae were kept in 2 l battery jars supplied with a slow stream of air bubbles for agitation. Water was changed on alternate days and *Dunaliella salina* was supplied at a concentration of about 10^4 cells ml^{-1} for food.

Microscopy

Catecholamine containing cells were visualized by the glyoxilic acid induced fluorescence technique described by Sharpe and Atkinson (1980). Larvae were placed in a solution containing 2% glyoxilic acid and 0.1 *M* phosphate buffer (pH 7.0, 4°C). After 3 to 5 min, larvae were placed on a glass slide and dried with a hair drier. The slides were heated to 100°C on a hot plate for 5 min before being mounted in liquid paraffin under a thin, glass cover slip. Specimens were examined immediately with a Zeiss Universal microscope fitted with UV epifluorescence equipment. An excitation filter with a peak transmittance of 364 nm was used on conjunction with a chromatic beam splitter (FT420) and a barrier filter (LP 418) reflecting light below 418 nm.

Larvae were fixed for electron microscopy in a solution of 2.5% glutaraldehyde in 0.12 *M* phosphate buffer, 0.14 *M* NaCl, and 0.2% tannic acid (Simionescu and Simionescu, 1976). Specimens were post fixed for 1 h at room temperature in 2% osmium tetroxide in 1.25% $NaHCO_3$. After dehydration by alcohol exchange, specimens for transmission electron microscopy (TEM) were infiltrated and embedded in Polybed (Polysciences Inc.). Sections were cut on a diamond knife and mounted on parlodion coated copper grids and stained with 50% ethanol saturated with uranyl acetate, followed by lead hydroxide chelated with sodium citrate. Observations and micrographs were made using a Philips EM300 electron microscope.

Electrical stimulation

Competent larvae were electrically stimulated after attachment to a fine tipped, polyethylene suction electrode (tip diameter 25–50 μm). Stimuli were from a Grass S44 square wave stimulator used in conjunction with a Grass SIU5 stimulus isolation unit. Threshold levels were determined by increasing the voltage output by 5 volt increments between 5 and 50 volts and by 25 volt increments to 150 volts. Stimuli were repetitive pulses of 5 ms duration delivered at a rate of 5 pulses per s for a total of 10 s. Preparations were allowed to stabilize for one min between treatments, and temperatures in the bath were maintained between 10° and 15°C.

Excisions and chemical stimulation

Portions of competent larvae were excised using tungsten needles prepared by etching in hot sodium nitrite. The reactions of excised parts and whole larvae to various chemicals was tested. Neurotransmitter and neuroinhibitor substances were initially prepared as 10^{-3} M stock solutions that were diluted immediately before use in 0.45 μm filtered sea water. A crude preparation of the natural cue to metamorphosis was made by stirring sand from an aquarium containing adult sand dollars in an equal volume of sea water for 5 min and then filtering the water. The crude preparation was tested for activity using 10 to 20 competent larvae. Only preparations that would induce greater than 90% metamorphosis within 5 min were used.

RESULTS

Larval nervous system

Glyoxilic acid induces a bright blue-white fluorescence of what are apparently nerves in the pluteus of *D. excentricus* (Fig. 1). These nerves are associated predominantly with the ciliary bands surrounding the arms and the larval mouth. Diffuse fluorescence associated with the adult rudiment and bright red fluorescence in the gut were considered artifactual. Fluorescence associated with ciliary bands appears as narrow tracts, 1–5 μm wide at the base of the ciliary epithelium (Figs. 1b, c, f). The tracts consist of axons less than 0.5 μm thick. The number of axons is greatest in regions of ciliary band between the larval arms and least within the arms themselves. Although there were some bright spots of fluorescence, 1–2 μm in diameter within the tracts, there were no obvious cell bodies.

A prominent oral ganglion is located within the adoral ciliary band (Fig. 1e). Forty to fifty brightly fluorescing cell bodies are dispersed along the lower lip of the larval mouth. These cells appear flask shaped, are 10 μm wide basally, and form a narrow neck, 2.5 μm wide, that extends apically up to 20 μm. The basal portions of the cells are associated with a neuropile from which numerous dendritic projections extend. The adoral ciliary band is organized in a manner similar to the ciliary bands of *Strongylocentrotus purpuratus* described by Burke (1978). Spindle shaped ciliated cells surround an accumulation of axons located basally in a central position. In the lower lip, the axons form a neuropile that is up to 15 μm in diameter and is a major portion of the band. The axons that make up the neuropile range from 0.3 μm to 0.6 μm in diameter (Figs. 2a, c). They contain mitochondria, microtubules, and numerous vesicles, 60–75 nm in diameter which frequently contain an electron dense core (Figs. 2c, d). Interspersed with the axons are verrucosities, 1.5 to 2 μm in diameter containing a dispersed flocculent material. The axons also contain vesicles that are frequently located adjacent to the plasmalemma (Figs. 2a, d). Occasionally, cells within the ciliated epithelium contributed a process to the axonal tract (Fig. 2b). These cells typically have a basally located nucleus, 5 μm in diameter, and a narrow neck extending toward the surface of the epithelium. The cytoplasm of these cells contains microtubules, clumped ribosomal material, and numerous vesicles up to 1 μm in diameter, containing course granular or flocculent material.

A region of neuropile, 80 μm by 50 μm is located between the anterolateral arms of the apical surface of the oral hood (Fig. 1d). Numerous axons form a swollen commissure between the axonal tracts of the ciliary bands. An overall diffuse brightness is associated with the neuropile, and some axons fluoresce more brightly than others, but no cell bodies were apparent. Ultrastructurally the apical neuropile is

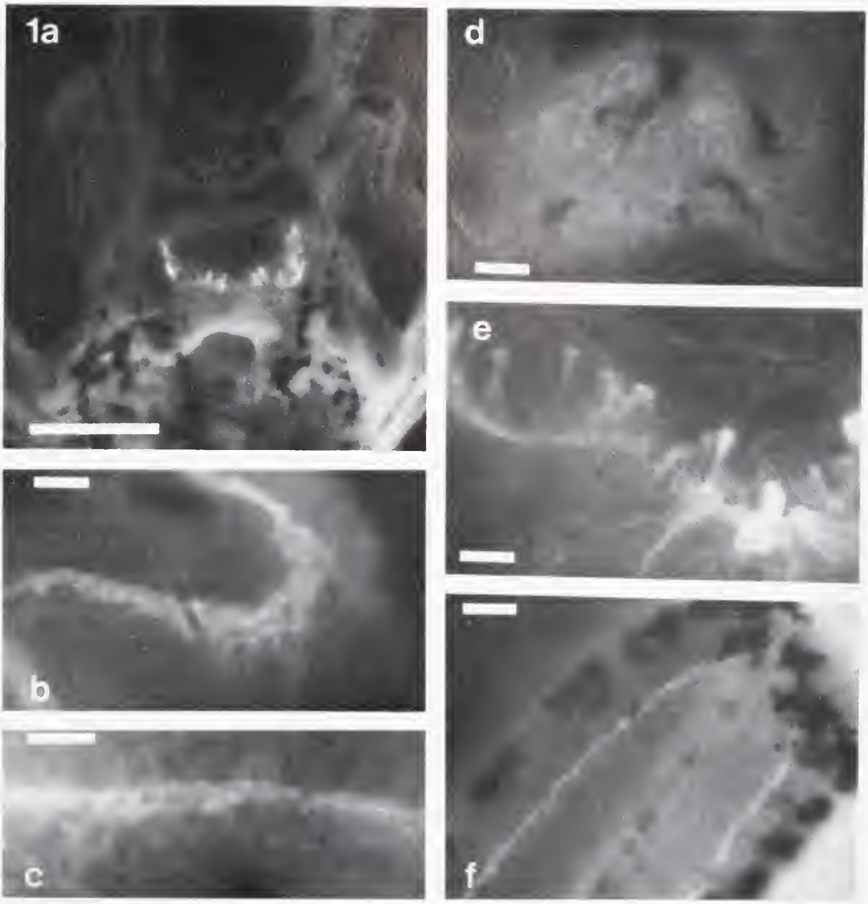

FIGURE 1 a. Competent larva of *Dendraster excentricus* prepared for glyoxilic acid induced fluorescence of catecholamines. Bar = 100 μm. b. Detail of a region of ciliary band between post-oral and posterodorsal arms of competent larva. Bar = 10 μm. c. Portion of pre-oral transverse ciliary band. Bar = 10 μm. d. Apical neuropile of competent larva, dark regions are pigment cells that overlie the neuropile. Bar = 10 μm. e. Detail of the oral ganglion. Bar = 10 μm. f. Tip of post-oral arm. Bar = 10 μm.

a plexus of axons and verrucosities similar to the axonal tract of the oral ganglion described above (Fig. 3). It is up to 20 μm thick and is covered by a 10 μm thick epithelium.

Electrical stimulation

Direct electrical stimulation induced immediate metamorphosis when a suction electrode was attached to either the oral ganglion or the apical neuropile (Table I, Fig. 4). Metamorphosis could also be induced when the electrode was attached to the oral surface of the adult rudiment or the ciliary band on the dorsal surface between the posterodorsal arms, but these locations were successful in less than half of the preparations attempted, and mean thresholds were higher than those needed for the oral ganglion or the preoral plexus. Stimulation with the electrode attached to the epidermis at the posterior end never induced metamorphosis.

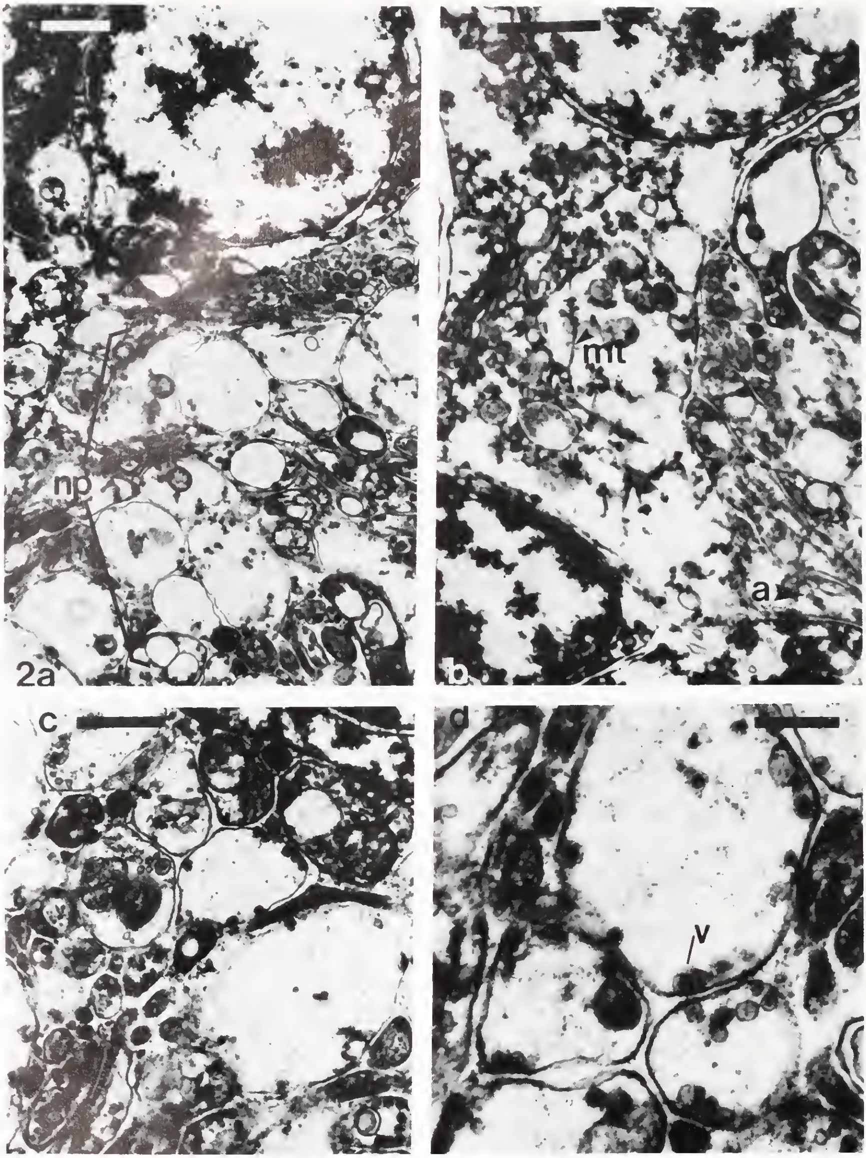

FIGURE 2 a. Transverse section through the oral ganglion of competent larva of *Dendraster excentricus*. Bar = 1 μm. b. Section through cell of epithelium overlying the neuropile which appears to contribute an axonal process (a) to the neuropile. Bar = 0.5 μm. c. Detail of neuropile from transverse section through it. Bar = 0.5 μm. d. Varrucosities of axons located within the neuropile region of the oral ganglion. Bar = 0.25 μm. a, axonal process; mt, microtubules; np, neuropile; v, vesicles.

Typically, when the electrode was attached to either the apical neuropile or the oral ganglion, stimuli of 1 to 5 volts evoked ciliary reversals and twitch responses from the larvae. Once the threshold was reached, the adult rudiment was activated, as indicated by movements of the spines and tube feet, and the entire oral hood flexed ventrally (Fig. 4b). Eversion of the adult rudiment followed within 1 to 2

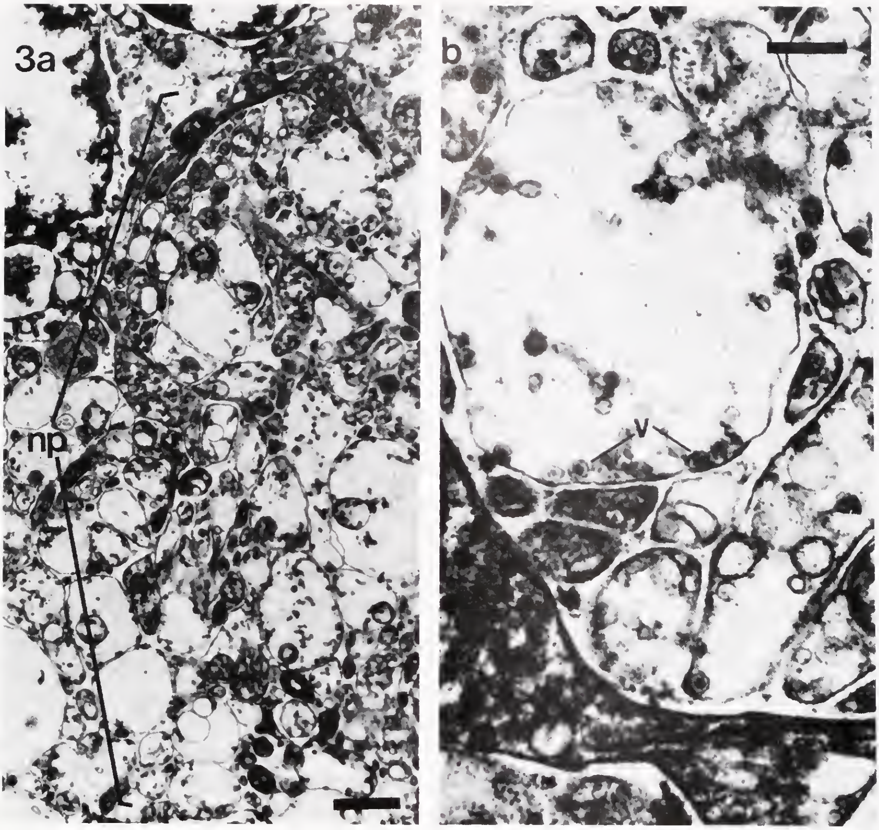

FIGURE 3 a. Transverse section through apical neuropile of competent larva of *Dendraster excentricus.* Bar = 1 μm. b. Detail of neuropile showing axonal profiles and varrucosites. Bar = 0.25 μm. np, neuropile; v, vesicles.

min, and the remainder of metamorphosis proceeded in typical sequence. Juveniles that were induced to metamorphose by electrical stimulation appeared normal and lived indefinitely.

When the electrode was attached to the ciliary band on the dorsal surface of the larva between the posteriodorsal arms, the oral surface of the adult rudiment, or

TABLE I

Summary of experiments correlating position of the electrode with the threshold of stimuli that will induce immediate metamorphosis of competent larvae of Dendraster excentricus

	Electrode location				
	Oral ganglion	Apical neuropile	Posterior end	Ciliary band	Oral surface of rudiment
Number metamorphosed	7/8	7/8	0/5	3/7	3/7
Mean threshold (Volts)	18.6 ± 6.3	22.9 ± 13.8	*	83.3 ± 28.9	83.3 ± 57.7

* Metamorphosis was never induced with electrode located here regardless of the intensity of stimulation.

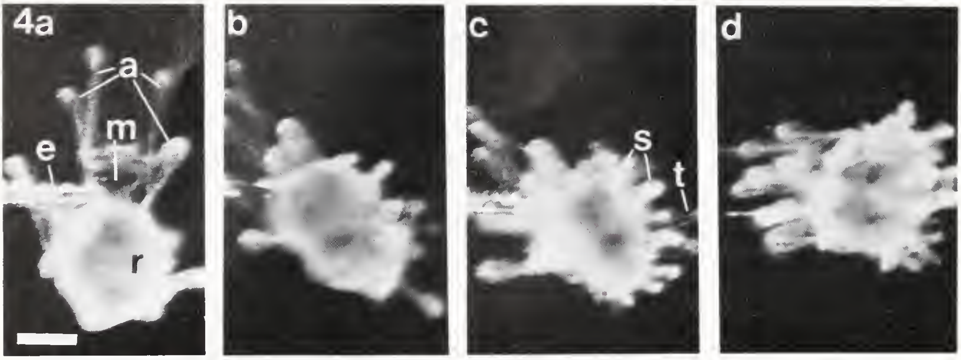

FIGURE 4. Competent larva of *Dendraster excentricus* attached to a fine-tipped, suction electrode in region adjacent to the oral ganglion. a. Prior to stimulation. b. 1 min after stimulation, pre-oral region has flexed anteriorly and the oral rudiment has activated and begun to evert. c. 4 min after stimulation, rudiment is fully everted and epithelium of larval arms has begun to retract. d. 10 min after stimulation, larva is no longer attached to the electrode and retraction of arm epithelium has been about half completed. Bar = 100 μm. a, arms; e, electrode; m, larval mouth; r, adult rudiment; s, spines on adult rudiment; t, tube feet.

the posterior end of the larva, responses such as ciliary reversals or twitches of larval muscles were not evoked until stimuli were 15 to 20 volts. If the threshold for metamorphosis was reached, larvae responded immediately in the same manner as described above. If the threshold was not reached, and the electrode was repositioned to the apical neuropile or the oral ganglion, metamorphosis could not be induced at any stimulation potential.

Excisions

Competent larvae can be dissected using fine tungsten needles. When the entire oral hood, including both the oral ganglion and the apical neuropile, was excised both halves underwent their characteristic reactions of metamorphosis (Table II, Figs. 5, 6). The adult rudiment was activated and everted within 15 min. A normal

TABLE II

Summary of experiments examining the effects of excisions of certain parts of the larval body on metamorphosis of competent larvae of Dendraster excentricus

Treatment	Number that underwent metamorphic Reaction*	
	Excised part	Part containing adult rudiment
Oral hood excised (includes oral ganglion and apical neuropile)	12/15	11/15
Preoral hood excised (includes only apical neuropile)	3/15	2/15
Preoral hood excised and subsequently treated with crude preparation of cue for metamorphosis (15 min)	11/12	10/12
Larval arms excised	0/20	0/20
Larval arms excised and subsequently treated with crude preparation of cue for metamorphosis (1 h)	0/20	19/20

* Metamorphic reactions described in text.

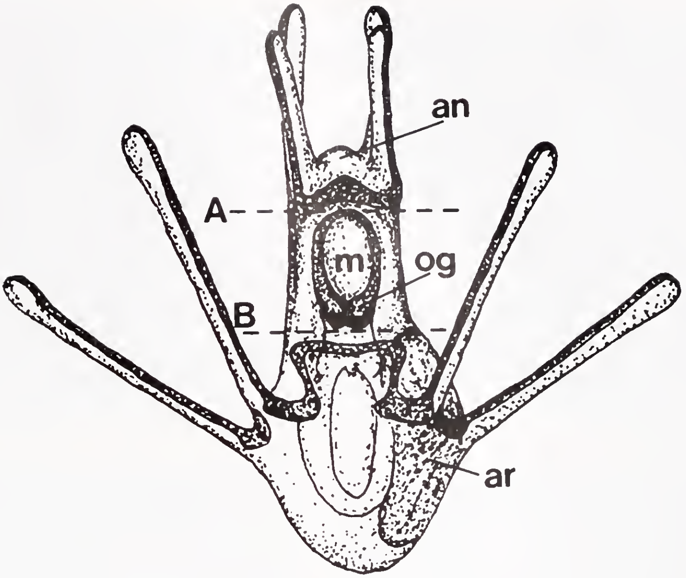

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FIGURE 5. Sketch of *Dendraster excentricus* larva showing levels at which cuts were made to excise preoral hood (A) and oral hood (B). an, apical neuropile, ar, adult rudiment; m, mouth; og, oral ganglion.

looking juvenile resulted. The oral hood's response was the sequence of contraction and histolysis that the larval epidermis typically undergoes during metamorphosis in intact larvae (Cameron and Hinegardner, 1978; Chia and Burke, 1978) (Fig. 6). In larvae in which only the pre-oral hood was removed so that the portion containing the adult rudiment also contained the tissues of the larval mouth and the oral ganglion, neither portion spontaneously metamorphosed within 1 h (Table II, Figs. 5, 6). When these two portions of the larva were exposed to a crude preparation of the cue that induces metamorphosis in intact larvae, both portions metamorphosed within 15 min. When individual larval arms were removed from larvae, the cilia continued to beat causing the arms to swim for at least 24 h (Table II). Arms treated with the crude preparation of the natural cue to metamorphosis also swam indefinitely showing no signs of the contraction-histolysis response of metamorphosis.

Neurotransmitters and neuroinhibitors

When larvae were put into SW containing neurotransmitter substances, metamorphosis was induced by dopamine, but with relatively low incidence (Table III). Excised larval arms responded to L-dopa and dopamine in a more consistent manner by undergoing the contraction-histolysis sequence of metamorphosis (Table IV).

To test the effects of catecholamine neuroinhibitors, larvae were exposed to the natural cue for metamorphosis in the presence of an inhibitor. Only 10^{-4} *M* reserpine appeared to interfere with the induction of metamorphosis; 6/18 metamorphosed within one hour, whereas those treated with the cue alone (19/20) and those treated with 10^{-4} isoproterenol (20/20) appeared unaffected. Lower concentrations of either drug also appeared to have no effect. Neither drug, at any of the concentrations attempted, had any effect on the induction of metamorphosis by electrical stimulation.

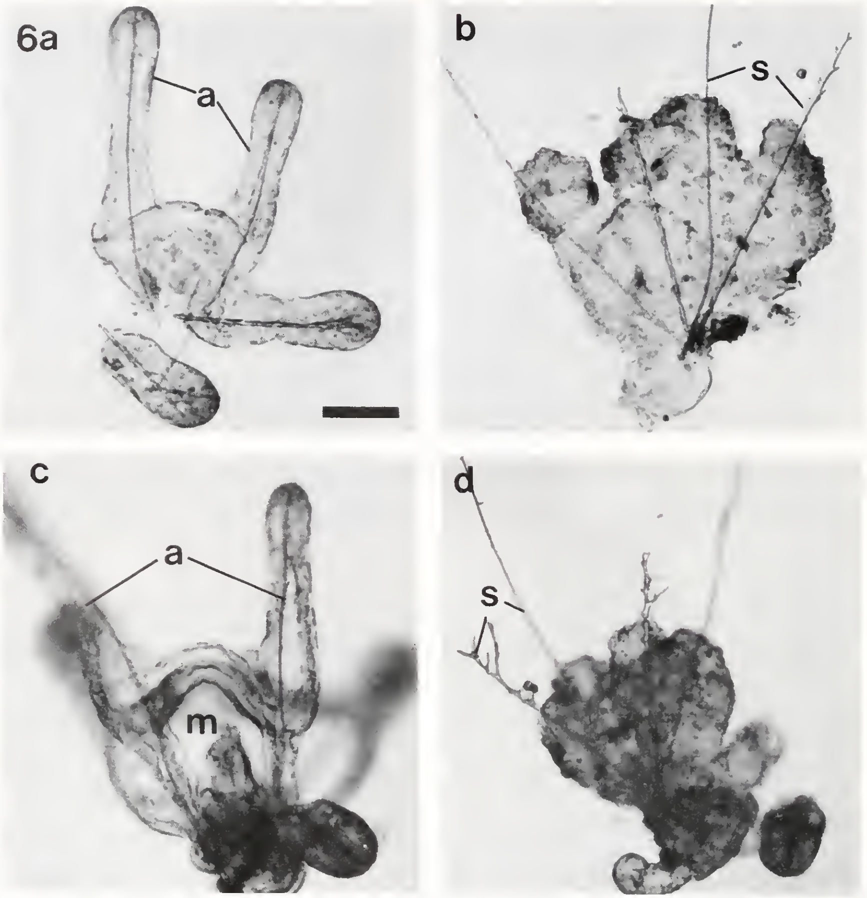

FIGURE 6. Excised pre-oral hood of competent larva of *Dendraster excentricus* after one h in sea water. b. Excised pre-oral hood after 30 min exposure to crude preparation of the cue for metamorphosis. c. Excised oral hood region. d. The same oral hood as is figured in c, after fifteen min in sea water. Bar = 100 μm. a, larval arms; m, larval mouth; s, larval skeleton.

DISCUSSION

Several structures in the echinopluteus have been suggested to be parts of a larval nervous system. MacBride (1914) identified the apical complex, a thickened epithelium on the oral hood, as a nervous structure. Mortensen (1920) suggested that the adoral ciliary band contains nerves. Gustafson (1969) and Ryberg (1977) propose that neuron-like cells spanning the blastocoel comprise a larval nervous system in *Psammechinus miliaris.* Burke (1978) described a system of nerve cells and axonal tracts associated with the ciliary bands and esophageal muscles in *Strongylocentrotus purpuratus.* The histochemical evidence presented here supports the idea that the nervous system consists of tracts of axons in the ciliary bands and corroborates, in part, the histochemical evidence presented by Ryberg (1974) for localization of biogenic amines in the ciliary bands and parts of the larval gut.

The structure of both apical and adoral neuropiles is similar to that of the radial nerves and nerve rings of adult echinoderms (Pentreath and Cobb, 1972). All of

TABLE III

Summary of experiments testing the effects of neurotransmitter substances on competent larvae of Dendraster excentricus

Neurotransmitter	Number metamorphosed (1 h)		
	10^{-3} *M*	10^{-4} *M*	10^{-5} *M*
Acetylcholine Chloride	—	—	—
5-Hydroxytryptamine	—	—	—
γ-Amino-n-butyric Acid	—	—	—
Epinephrine	—	—	—
Noradrenaline	—	—	—
3-Hydroxytyramine (Dopamine)	5/20	1/20	5/20
L-β-3,4-Dihydroxyphenyulalanine (L-DOPA)	—	—	—

these structures consist of tracts of relatively densely packed nerve fibers aligned longitudinally. Nerve cell bodies are only rarely encountered within these regions (Cobb, 1970; Pentreath and Cobb, 1972). The radial nerve cords and nerve rings of adult echinoderms have been ascribed the role of a central nervous system (Smith, 1965; Pentreath and Cobb, 1972). Similar accumulations of axons may function as coordination and integration centers within the larval nervous system. The neuropile associated with the lower lip of larvae is surrounded by nerve cell bodies, so the structure has been termed a ganglion. The connection of these cell bodies with the external surface suggests that they may play a sensory role.

Experiments in which the position of the stimulating electrode was varied show that when the stimulus is delivered to regions associated with either the apical neuropile or the oral ganglion, metamorphosis is more reliably induced and the mean threshold of stimulation is less than at other locations. It is proposed that metamorphosis results because of direct stimulation of the nerves in these regions. Elements of the nervous system are associated with all of the other sites of stimulation, but the apical neuropile and oral ganglion appear to be most effective in inducing metamorphosis. Cameron and Hinegardner (1974) reported that *Arbacia punctulata* larvae metamorphosed in response to electrical stimulation of 150 V delivered in 1 ms pulses. They did not note any specific locations for the placement of the electrode, but as results reported here indicate, electrode placement is not critical with high voltage stimuli. Higher voltage stimuli may cause tissue damage,

TABLE IV

Summary of experiments testing the effects of neurotransmitter substances on excised larval arms

Neurotransmitter	Number undergoing metamorphic reaction (1 h)		
	10^{-4} *M*	10^{-5} *M*	10^{-6} *M*
Acetylcholine Chloride	—	—	—
5-Hydroxytryptamine	—	—	—
γ-Amino-n-butyric Acid	—	—	—
Epinephrine	—	—	—
Noradrenaline	—	—	—
3-Hydroxytyramine (Dopamine)	4/6	6/6	—
L-β-3,4-Dihydroxyphenyulalanine (L-DOPA)	2/6	6/6	—

as larvae for which a threshold was never reached did not metamorphose when the electrode was repositioned.

The excision experiments demonstrate interactions between tissues of the oral hood and the remainder of the larva containing the adult rudiment. Because both halves of larvae dissected below the larval mouth will spontaneously metamorphose, it is proposed that there is a mutual inhibitory control between these portions of the larva. Since excision of the pre-oral region does not induce spontaneous metamorphosis, it is deduced that inhibitory control is localized in the region of the larval mouth.

The excised pre-oral region of the larva and the remaining portion of the larva containing the oral regions and the adult rudiment will only undergo metamorphic reactions in the presence of the natural cue for metamorphosis. This suggests that both portions contain receptors for the cue and the necessary mechanisms for transmitting this stimulus to all the tissues involved in the metamorphic response. Because tissues of the larval arms are apparently unable to perceive the cue, or initiate the contraction and histolysis response, it is proposed that a stimulatory center is localized in the pre-oral region of the larva. A second stimulatory center would have to be located in the portion of the larva containing the oral regions and the adult rudiment.

Cameron and Hinegardner (1974) showed that larvae of *Arbacia punctulata* are induced to metamorphose by a combination of a soluble chemical cue and tactile stimulation of the primary podia of the adult rudiment. Burke (1980) has suggested that sensory receptors on the tips of the primary podia are involved in the perception of these cues in several species of regular echinoid. *D. excentricus* differs from *A. punctata* in that larvae of the former respond to a chemical cue alone, and the adult rudiment remains inactive prior to the initiation of metamorphosis. Although *D. excentricus* possess similar podial sensory receptors (Burke, unpublished observations), experiments reported here indicate that the receptors that receive the cue may be more dispersed. Although the excision experiments indicate that there is a stimulatory center located on the pre-oral hood and another in the regions posterior to it, it does not definitely indicate that these centers correspond to the locations of receptors. However, receptors must be located somewhere on the pre-oral hood.

Glyoxilic acid induced fluorescence has been suggested to be specific for dopaminergic nerve cells (Grace and Bunny, 1980; Sharpe and Atkinson, 1980), however Keenan and Koopowitz (1981) have presented evidence that glyoxilic acid will induce fluorescence of L-dopa, L-dopamine, L-tyrosine, and norepinephrine and emittence spectra for all these catecholamines are indistinguishable. The observation of vesicles with an electron dense core, similar to those observed in catecholamine containing nerves in adult echinoderms (Pentreath and Cobb, 1972), also supports the hypothesis that larval nerves contain catecholamines.

The inconsistent response of whole larvae to dopamine is difficult to interpret, though it does suggest dopamine may be involved in some stage of the induction of metamorphosis. The responses of isolated larval arms were more consistent, though relatively high doses for neurotransmitters were necessary to bring about a response. These observations are compatible with the hypothesis that either L-dopa, dopamine, or a derivative of them acts as a chemical messenger during the induction of metamorphosis. This endogenous chemical signal may act directly on the larval and adult tissues that respond during metamorphosis, or it may act indirectly by causing the release of additional substances that stimulate the tissues containing the effectors of metamorphosis. The fact that reserpine, a substance thought to deplete catecholamines in vertebrate tissues (Goodman and Gilman, 1970), interferes with the induction of metamorphosis supports this hypothesis. As with all experiments in which substances are added to a sea water bath, effective dose and site of action are impossible to determine.

The three principal observations of this research are: 1) electrical stimulation of nerve centers induces metamorphosis, 2) stimulatory and inhibitory control is associated with the same regions of the larval nervous system, and 3) neurotransmitters can induce metamorphic responses in isolated tissues. These results support the proposal that the nervous system controls metamorphosis. Observations and experiments also indicate that control resides in the apical neuropile and the oral ganglion. It is tentatively suggested that a cue for metamorphosis from the adult environment is received by receptors that communicate with the larval nervous system. Nerve centers release inhibitory control and stimulate tissues to initiate the sequence of developmental events of metamorphosis. The mechanisms of inhibition and stimulation have not been determined. However, the nervous system has not been observed to make axonal connection with all the tissues that respond during metamorphosis, indicating that an alternative mechanism may be involved.

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EGGS OF *PALAEMON MACRODACTYLUS:* I. ATTACHMENT TO THE PLEOPODS AND FORMATION OF THE OUTER INVESTMENT COAT

WILLIAM S. FISHER AND WALLIS H. CLARK, JR.

Aquaculture Program, Animal Science Department, University of California, Davis, CA 95616

ABSTRACT

Eggs of the estuarine shrimp, *Palaemon macrodactylus,* were examined for their method of attachment during the brooding period. They were attached to each other and to setae of the maternal pleopods by an externally applied outer investment coat that is believed to originate within the pleopods. A precursor substance was stored in packets extending longitudinally along the basipodites of the female pleopods, apparently originating in specialized epithelial cells continuous with the packets. At ecdysis prior to oviposition, the packets became depleted and the attachment or adhesive material became evident externally, conforming to the external surfaces of the pleopods. This occurred in the presence and absence of mature eggs in the ovary. If mature eggs were present, they were soon (within one-half day) extruded into the incubation chamber, coated with the adhesive material, and attached. Both fertilized and unfertilized eggs attached in the same manner, although unfertilized eggs were either shed or removed by the female within a few days. Eggs from pleopod-excised females did not attach, had no outer investment coat, and deteriorated rapidly. This attachment mechanism differs from those described for other decapods.

INTRODUCTION

In certain decapod crustaceans, the females incubate their embryos on pleopods (swimmerettes) on the abdomen until hatching. During this period, the embryo's investment coats (egg coats, egg envelopes) protect it from physical and chemical stresses and maintain the internal milieu. The outer investment coat, due to its immediate exposure to the aquatic environment, is of primary importance in this role. The outer coat has also been associated with the attachment of eggs to the maternal pleopods, selective permeability (Yonge, 1937), and osmotic hatching (Davis, 1965), and it may serve as a substratum for aquatic microorganisms (Johnson *et al.*, 1971). Several investigators, often using different species of brooding decapods, have presented conflicting theories concerning the origin and formation of this important protective layer. It is the intent of this study to examine the outer investment coat of the estuarine shrimp *Palaemon macrodactylus,* and compare its egg attachment process with that described for other species. The word "egg" is often used in the general sense to identify either unfertilized eggs, fertilized eggs, or both. When necessary, these will be specifically designated as "fertilized" or "unfertilized." Fertilized eggs are also appropriately termed "embryos."

An early theory of attachment (Yonge, 1937) was based on work with the macruran *Homarus vulgaris,* which was found to have glands in the female pleopods that secreted a material to coat the newly extruded eggs. This material simultaneously formed the outer investment coat and attached the eggs to the pleopodal setae. Yonge's hypothesis explained Andrews' (1906) observations, which reported the

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secretion of a mucous substance into the incubation chamber of crayfish just prior to egg extrusion. In contrast, Burkenroad (1947) felt the outer coat from eggs of the caridean *Palaemonetes vulgaris,* was formed by the newly extruded eggs upon contact with a medium such as sea water. He believed the outer coat was inherently capable of fusing with other eggs or maternal setae for attachment, but in some cases required an "intensifier substance" such as the material released by the pleopodal glands of the macrurans.

Another contrasting theory was presented by Cheung (1966) who found three embryonic coats for the brachyuran *Carcinus maenas.* All were formed by the ovum as a consequence of fertilization. Moreover, Cheung found no pleopodal "cement glands" associated with oviposition, a result that conflicted with reports on other decapods (Andrews, 1906; Yonge, 1937; Lloyd and Yonge, 1940; Stephens, 1952). The absence of pleopodal glands coupled with exclusive egg-to-seta attachment also contradicted the self-fusibility theory of Burkenroad (1947). Yet recent ultrastructural evidence from *C. maenas* (Goudeau and Lachaise, 1980) and from the spider crab *Libinia emarginata,* (Hinsch, 1971) supports Cheung's concept that the outer investment coat was formed from the vitelline envelope.

Cheung cited Hoglund (1943) and Jefferies (1964) to support his claim that carideans also required fertilization for egg attachment. Both investigators found that unfertilized eggs of shrimp would be extruded into the brood chamber, but they would not have an outer investment coat and would either not attach or would be shed within hours. Not cited by Cheung (1966), however, was the more common occurrence described by Jefferies (1964) of unfertilized eggs attaching for up to five days. Burkenroad (1947) also found that unfertilized *P. vulgaris* eggs became attached but, rather than being shed, were removed by the female within a few days. Unfertilized eggs remained attached when the female pereiopods were amputated, thus negating fertilization as a prerequisite for attachment. The ability to attach unfertilized eggs has also been noted for another Palaemonidae, *Macrobrachium rosenbergii* (Bardach *et al.,* 1972), and is described in this paper for an estuarine shrimp, *Palaemon macrodactylus.*

The reproductive pattern of *P. macrodactylus* is similar to that described for *M. rosenbergii* (Bardach *et al.,* 1972) and *Leander squilla* (Hoglund, 1943). Within hours after the female molts, the male deposits spermatophores externally on her ventral thorax. Eggs are extruded within 2–3 hours of mating, fertilized, and attached to pleopodal setae in the incubation chamber. *P. macrodactylus* was particularly well-suited for these studies because of its capacity to extrude eggs nearly every two weeks from April to October.

MATERIALS AND METHODS

Specimens of *P. macrodactylus* were collected from the Petaluma River in northern California and maintained from April to October in individual compartments in a well-aerated, closed circulating system at the University of California, Davis campus. Animals were kept at 21 to 25°C and 10 to 20 g/l salinity and were fed *Artemia salina* or frozen bay shrimp. Molting and egg extrusion were monitored daily. Animals were mated by placing a male in a female's compartment a few days before her expected molt. Egg extrusion and attachment took place for both mated and unmated females.

Eggs were obtained for microscopy directly from the ovary (unextruded), as attached unfertilized eggs (from unmated females), as attached fertilized eggs (from mated females), and as extruded but unattached eggs collected by excising the first or second pair of maternal pleopods before extrusion. This last procedure caused the eggs to fall directly from the oviduct to the floor of the aquarium. Samples of eggs and excised pleopods from animals in different stages of the reproductive cycle were fixed in 2% glutaraldehyde in a standard 0.1 *M* phosphate buffer at pH 7.3. Samples were osmified (0.1% OsO_4), embedded in a low viscosity epoxy resin (Spurr, 1969) and sectioned on a Porter Blum MT-2 ultramicrotome. Thick sections (0.5 μm) were stained 10–15 seconds with borate buffered toluidine blue (Dewel and Clark, 1972). Fixed tissues were also embedded in butoxyethanol/glycol methacrylate medium (Scientific Chemical Co., Huntington Beach, CA.) and stained with periodic acid-Schiff's (PAS) reagent (Lillie, 1965). Samples were viewed with compound light microscopy.

RESULTS

During this study, female *P. macrodactylus* held in the system molted every 16.3 (±3.5 days), and eggs were extruded and attached to the pleopods after 76.5% of these molts, usually within 1 day. Unfertilized eggs were lost or removed from the pleopods after 1 to 3 days, while embryos remained attached for an average 14.3 (±0.8) day incubation period. Eggs teased from the ovary appeared to have a material on their surfaces that would form a coagulum on contact with water from the holding system. This coagulation caused the eggs to adhere to each other in a clump, but the clump could be teased apart easily with forceps.

During oviposition, the female stood upright and the eggs moved into a chamber formed by the pleopods and the lateral epimera (pleura) on the underside of her abdomen (Fig. 1). Eggs were attached to each other (Fig. 2) and to pleopodal setae by a connecting or adhesive material that formed the outer investment coat (Fig. 3). This occurred for both fertilized and unfertilized eggs. At sites of attachment, the adhesive material took the shape of a flattened strand ("connectant," Fig. 4) or a twisted stalk ("funiculus," Fig. 5).

Cross sections of mature eggs taken from the ovary had a single investment coat (the vitelline envelope) that appeared separated from the egg, probably caused during fixation (Fig. 6). Naturally spawned eggs that were attached to pleopods had an additional (outer) investment coat (Fig. 7). This external coat was PAS-positive, was of variable thickness, and connected the eggs to each other and to the pleopods; as such, it was the adhesive material previously described. Eggs not allowed to attach (from pleopod-excised females) resembled ovarian eggs in lacking the outer coat (Fig. 8). Also like ovarian eggs, these unattached eggs adhered to one another in water and could be teased apart easily. Such eggs deteriorated within 2 days; only the vitelline envelope remained, devoid of ooplasm and appearing as a "ghost capsule" (Fig. 9). This deterioration did not occur when normally attached eggs, fertile or infertile, were detached from the maternal pleopods.

Pleopods were examined to determine the origin of the adhesive material that formed the outer coat of the eggs. Moving distally, each pleopod (Fig. 10) is joined to the abdominal sternite by the precoxae and consists of a joint called the coxapodite, a long basipidite, an endopodite, and an exopodite. The posterior face of the basipodite is concave and, starting at the proximal end, has a central channel extending one-third to one-half the length of the basipodite (Fig. 11). A cross-section of the basipodite from a female with attached eggs (Fig. 12) shows the adhesive material intimately associated with the recesses of the channel. The adhesive material conforms to the external surface of the pleopod and wraps around pleopodal setae and the extruded eggs, thereby forming the outer investment coat. Figure 13 shows

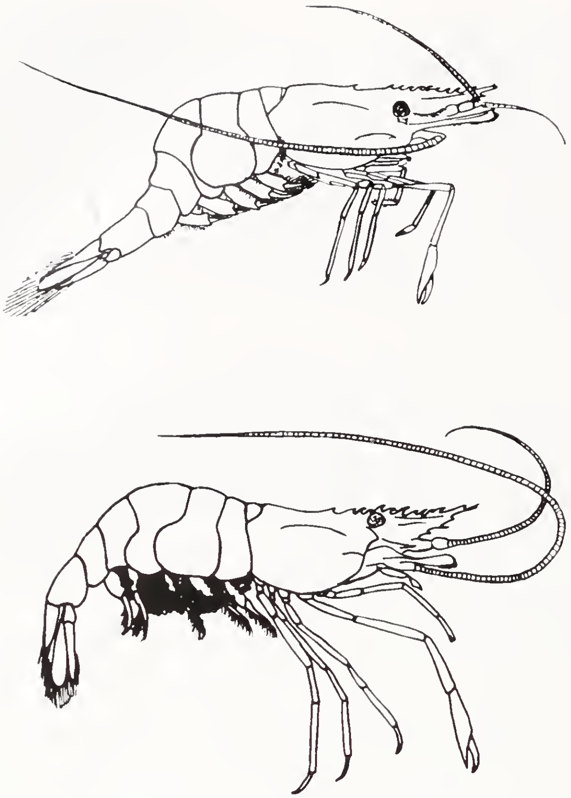

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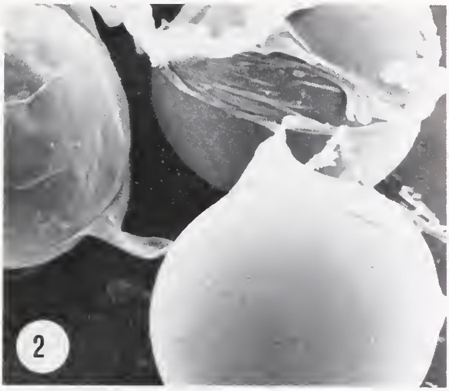
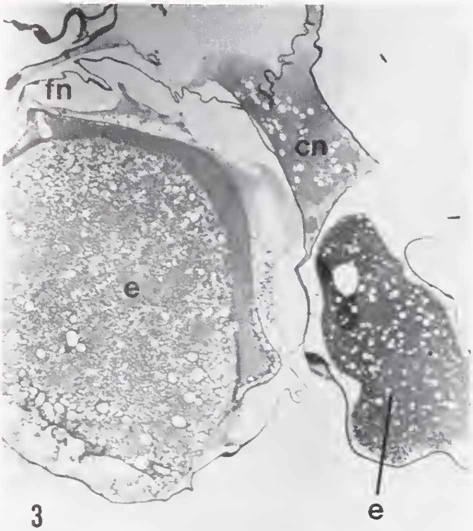
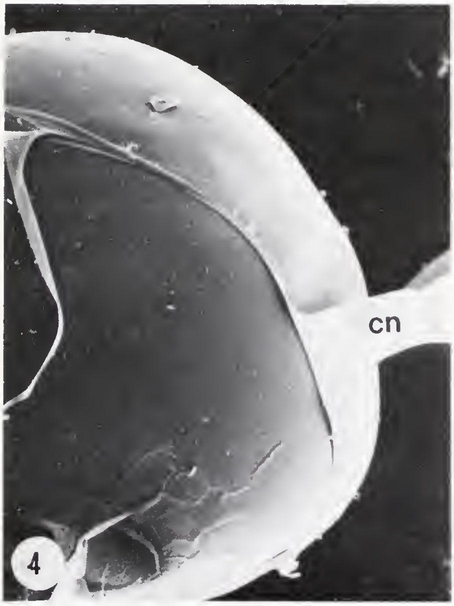
FIGURE 1. Top; illustration of a female *Palaemon macrodactylus*, extruding eggs from the oviduct into a chamber formed by the lateral plates (epimura) of the abdomen and the anterior extensions of the pleopods. Bottom; the extruded eggs are attached to the setae of the pleopods for a two-week brooding period.

this material enveloping a well-developed embryo. Adhesive material appeared at ecdysis prior to egg extrusion, but its presence was not necessarily related to extrusion since it appeared at ecdysis even when no ova were in the ovary.

Although the adhesive material was externally associated with the central channel of the basipodite, no ducts or direct portals from the interior of the pleopod were observed along the length of the channel. There were, however, long tubules or packets that contained slightly PAS-positive material. These packets (designated mucilage packets) appeared continuous with specialized epithelial cells (mucilage cells) found only in the coxapodite (Figs. 14, 15). The packets extended longitudinally into the basipodite (Fig. 16) and, proceeding distally, decreased in number until none could be found. Mucilage packets occurred in anterior, posterior, and median regions of the pleopod and, other than their proximal continuity with mucilage cells, showed no particular association with a cell or tissue type (Fig. 17). Mucilage packets were found prior to ecdysis (Fig. 18), but were absent or depleted immediately afterwards, coinciding with the appearance of adhesive material on the external surfaces of the pleopods (Fig. 19).







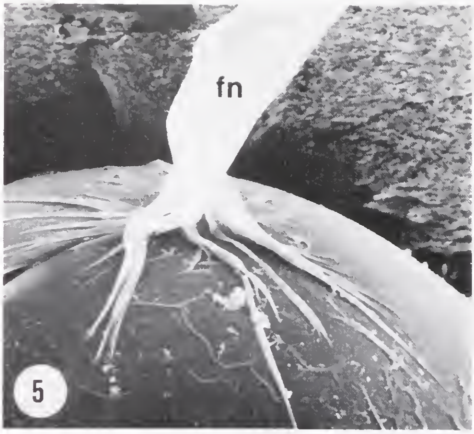


FIGURE 2. Scanning electron micrograph of eggs attached to one another by adhesive material. 80×.

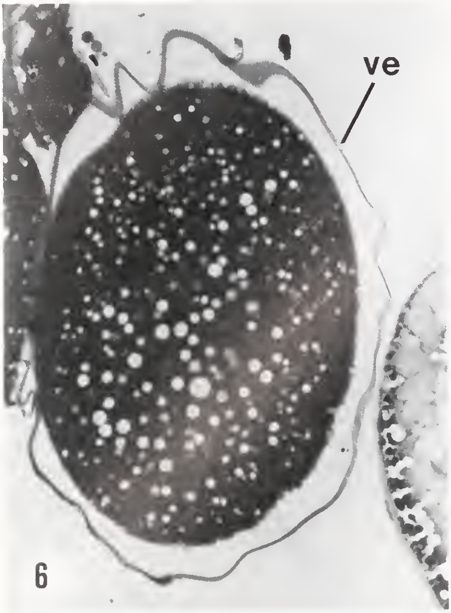
FIGURE 3. Cross-section of unfertilized eggs (e), showing adhesive material in the form of a connectant (cn) to other eggs or a funiculus (fn) to pleopodal setae. PAS-stain, 135×.

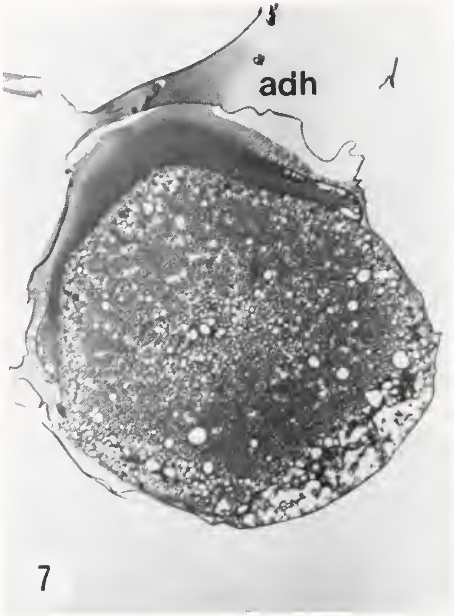
FIGURE 4. Scanning electron micrograph of adhesive material in the form of a connectant. 175×.

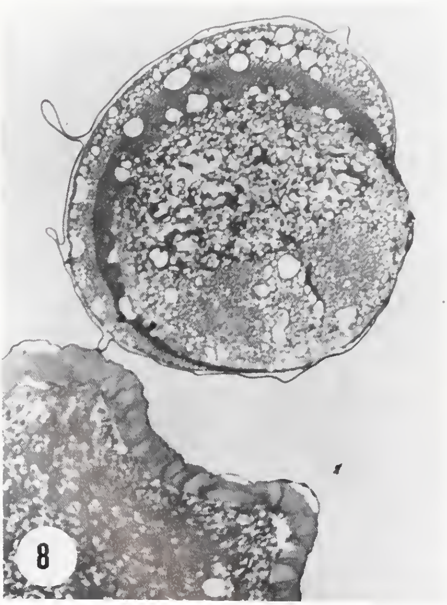
FIGURE 5. Scanning electron micrograph of adhesive material in the form of a funiculus. 320×.

DISCUSSION

The externally brooding caridean *Palaemon macrodactylus* exhibited a mechanism of egg attachment that differs from accounts of macruran and brachyuran egg attachment. A substance produced and stored in the female pleopods appeared to be released at molt to coat the external surfaces of the pleopods. Extruding eggs, fertilized or unfertilized, were connected to the pleopodal setae and to each other by the adhesive material, which simultaneously formed the outer investment coat of the eggs. This mechanism is unlike that suggested for *Homarus* (Yonge, 1937)







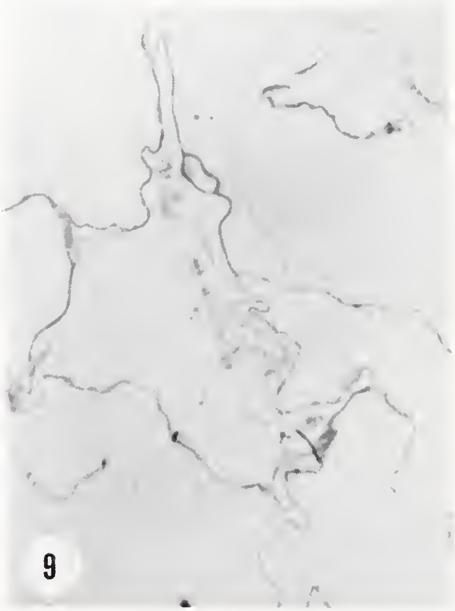
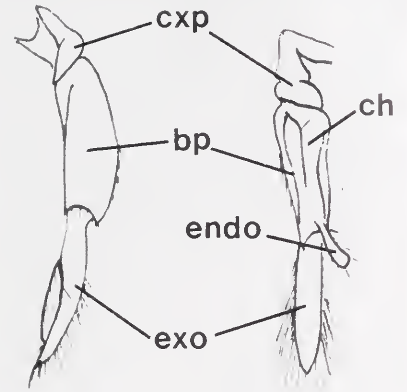


FIGURE 6. An ovarian egg showing the vitelline envelope (ve) which has been artificially elevated from the oolemma by fixation. Toluidine blue stain, 220×.

FIGURE 7. A naturally spawned, unfertilized egg detached from a maternal pleopod. The adhesive material (adh) forms the outer investment coat of the egg. PAS-stain, 180×.

FIGURE 8. Eggs from a pleopod-excised female extruded without becoming attached to maternal pleopods; they showed no sign of the adhesive material that forms the outer investment coat of naturally spawned eggs. PAS-stain, 145×.

FIGURE 9. Deterioration of eggs that were never attached (such as those in Fig. 8) after 2 days; only "ghost capsules" remain. PAS-stain, 170×.



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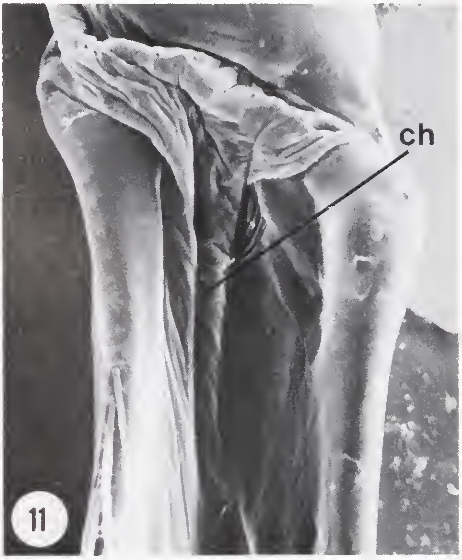

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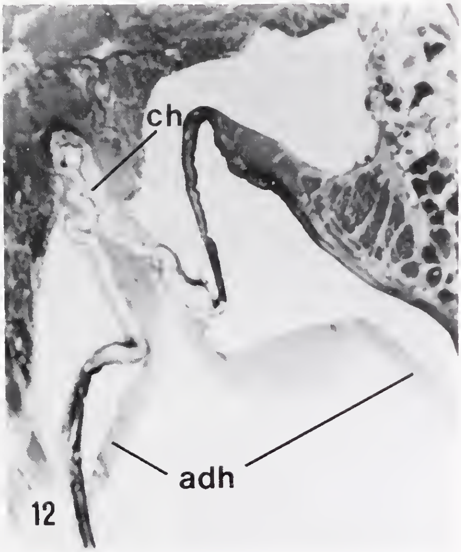

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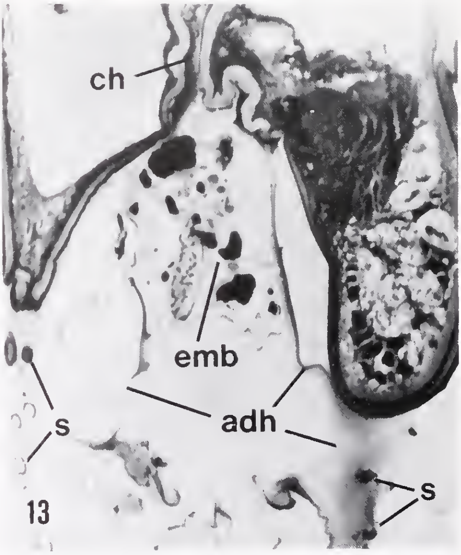

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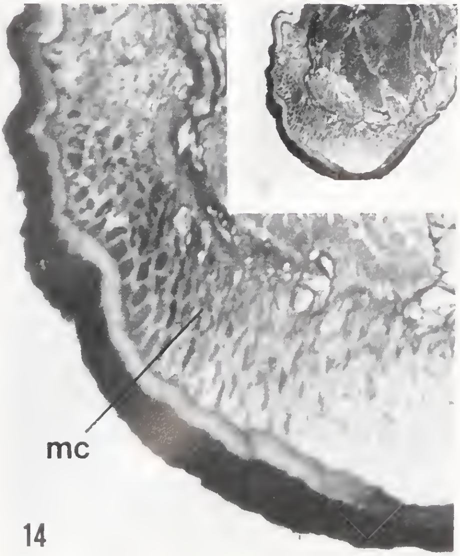
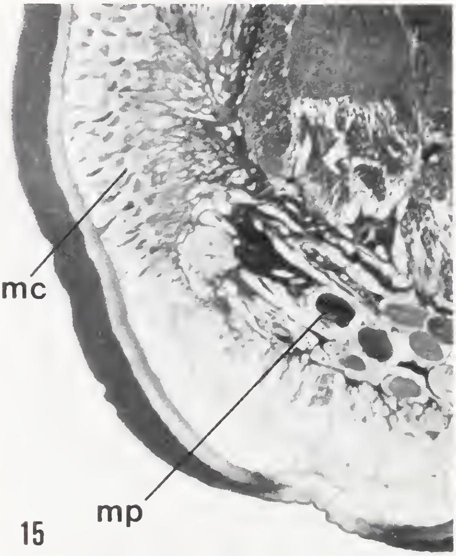
FIGURE 10. Illustrations of a pleopod from *P. macrodactylus*. Left; a right-side view, and right, a rear view. The pleopod consists of a coxapodite (cxp), a basipodite (bp), an endopodite (endo) and an exopodite (exo). A channel (ch) is found on the concave, posterior face of the basipodite. Eggs attach primarily to special setae (not shown) on the basipodites of brooding females.

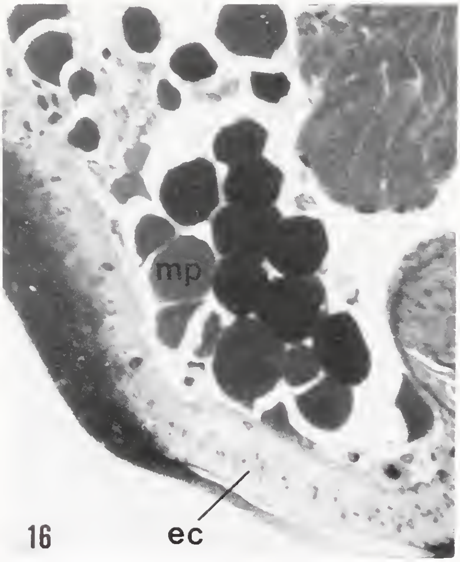
FIGURE 11. A scanning electron micrograph of the posterior face of a pleopod from a nonbrooding female, showing the central channel (ch) extending partway down the basipodite. 40×.

FIGURE 12. A cross-section through the proximal portion of the basipodite of a pleopod from an ovigerous (brooding) female shows the central channel (ch) and its intimate association with the external adhesive material (adh). Toluidine blue stain, 140×.

FIGURE 13. This cross-section through the basipodite of an ovigerous female shows an embryo (emb) lodged in the posterior concavity near the central channel (ch). The adhesive material (adh) encompasses the embryo and the pleopodal setae(s). Toluidine blue stain, 125×.







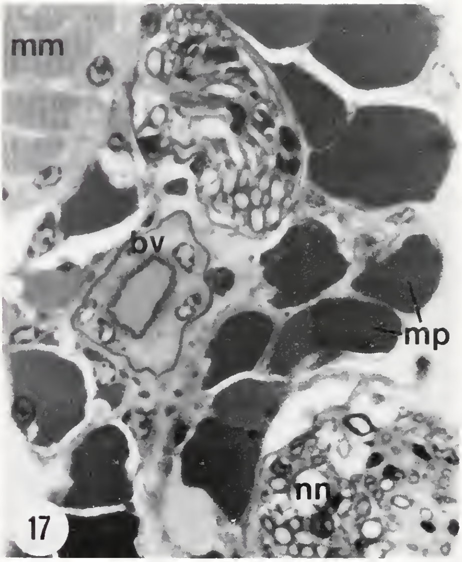


FIGURE 14. A cross-section through the coxapodite region shows the proximal portions of specialized epithelial cells, or mucilage cells (mc), that are believed to secrete material into mucilage packets (mp) for storage. Toluidine blue stain, 440×. (Inset shows the entire cross-section, 95×.)

FIGURE 15. The same pleopod (as shown in Fig. 14), sectioned a few microns distal, shows mucilage cells (mc) continuous with mucilage packets (mp), which contain a dark-staining material which is believed to be the precursor to adhesive material. Toluidine blue stain, 325×.

FIGURE 16. A cross-section of the same pleopod near the basipodite, shows the mucilage packets (mp) are not continuous with the more distal epithelial cells (ec) which elongate as the animal nears ecdysis. Toluidine blue stain, 300×.

FIGURE 17. In this cross-section of a different pleopod, mucilage packets (mp) are found in the center alongside muscle tissue (mm), blood vessels (bv) and nervous tissue (nn). Toluidine blue stain, 875×.

in that cement glands or ducts were not observed in *P. macrodactylus,* and secretion of adhesive material occurred before, rather than during, oviposition. It also differs from that proposed for *Carcinus* (Cheung, 1966) in that fertilization was not necessary for attachment and the outer layer was formed by material secreted from the female pleopods, not from individual eggs. Attachment in *Palaemonetes,* described by Burkenroad (1947) and Jefferies (1964) was probably the same as that described here for *P. macrodactylus,* but the adhesive material escaped detection because of its close conformity with the external surfaces of the pleopods.

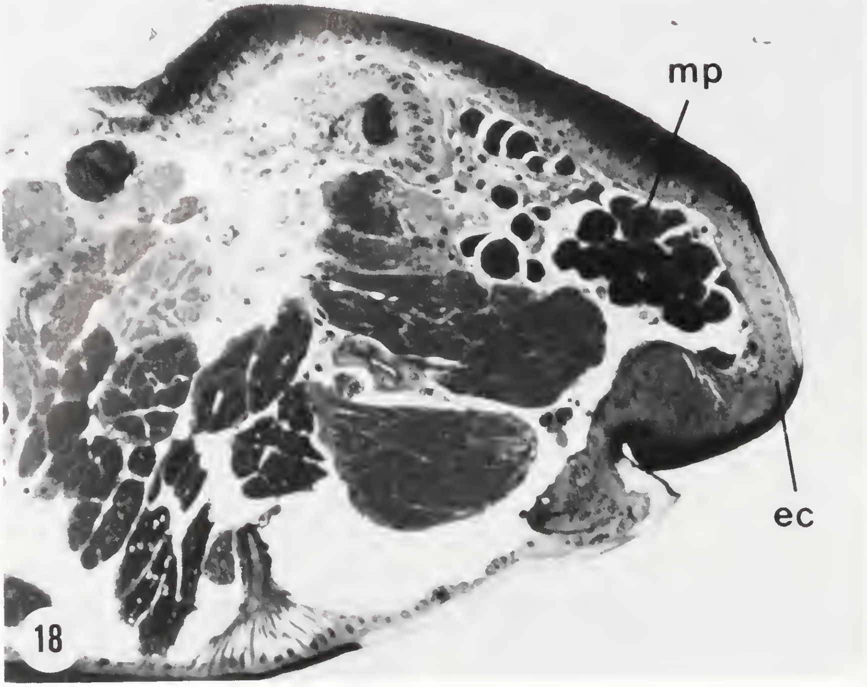
Eggs that were never attached (artificially produced by excision of the pleopods prior to extrusion) were not wrapped in this outer layer of material and, although they were slightly adherant, did not form connectants or strongly attach to each other. Clearly, the adhesive material had a major role in egg attachment. This externally applied layer also appeared to be responsible for protection of the inner coat (fertilization membrane for embryos, vitelline envelope for unfertilized eggs) and oolemma since unattached eggs deteriorated and became completely devoid of ooplasm within two days. A high number of bacteria inhabited the surfaces of unfertilized eggs without outer coats (Fisher, 1983) and may have obtained nutriment from escaping ooplasm or from the vitelline envelope.

It is not clear how the adhesive material is released, but it may be associated with the ecdysial process since it occurs at ecdysis rather than at oviposition. "Molting fluid" is believed to appear in the exuvial space (between the cuticle and the epidermal cells) in molting crustaceans (Kugler and Birkner, 1948; Needham, 1954) and in insects (Zacharuk, 1976) where resorptive enzymes in the fluid are secreted from the epidermal cells. Also in insects, Wigglesworth (1947) suggested that the outer layer of the exoskeleton is covered by a wax that is secreted by the epidermal cells immediately prior to ecdysis. In *P. macrodactylus,* the mucilage packets may secrete the stored mucilage into the exuvial space sometime between apolysis and ecdysis. After the exuvia is shed, the adhesive material coating the surfaces of the pleopods, as seen in Figures 13 and 19, is exposed.

Secretion can occur whether or not there are mature eggs in the ovary, implying that secretory activity is not regulated by an ovarian cycle. This is not without precedence; Stephens (1952) reached a similar conclusion concerning the activity of tegumental glands in the crayfish *Cambarus.* Cement glands in crayfish (Andrews, 1906; Stephens, 1952) and lobsters (Yonge, 1937) secrete at oviposition, a function not necessarily associated with molting events. In the Palaemonidae however, molting, mating and brooding are strictly sequential.

Although the importance of an externally applied outer investment coat has been stressed here, the role of the inner investment coat (fertilization membrane or vitelline envelope) should not be overlooked. Burkenroad's (1947) belief that the vitelline envelope had inherent fusibility properties is supported by the apparent coagulation on the surface of the vitelline envelope and the limited adherance of *P. macrodactylus* eggs teased from the ovary. Interaction between this coagulum and the externally applied adhesive material is very likely and may actually combine to form the outer investment coat. Concepts that considered pleopodal secretions as an "intensifier substance" (Burkenroad, 1947) or an investment-coat hardener (Cheung, 1966) may have been modified, but not disproven by the results of this study.

An acceptable general theory of decapod egg attachment is lacking, although several have been proposed. Yonge (1937) felt that all decapods would attach eggs in a manner similar to his description for *Homarus.* Hoglund (1943) also accepted this view. Cheung (1966) however, believed that his work with *Carcinus* established


mp
ec
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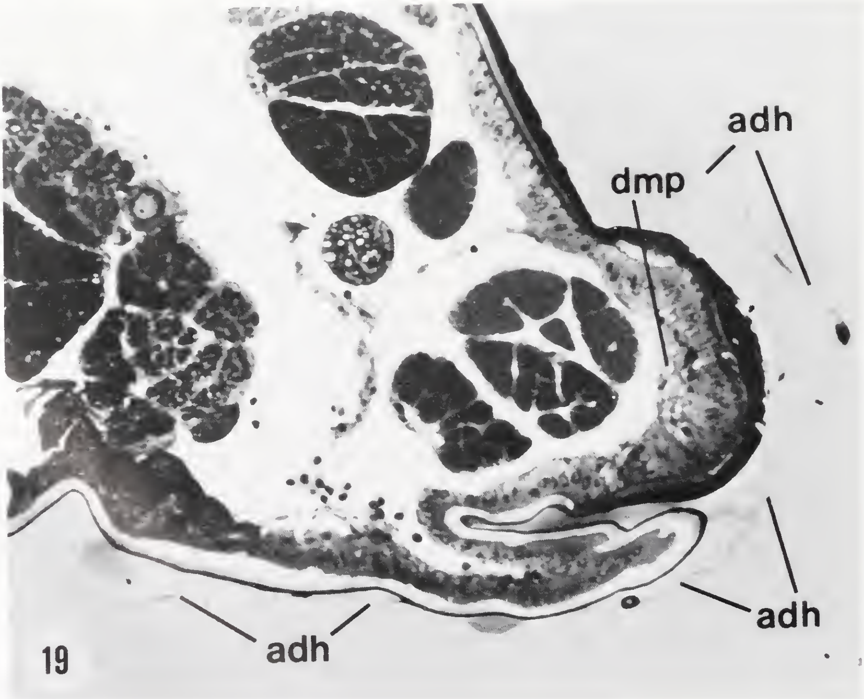
adh
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the patterns followed by all brooding decapods. Under this assumption, Cheung (1966) incorrectly interchanged experimental results from *Carcinus* with histological results from *Astacus*. And although Cheung (1966) examined the macrurans *Nephrops* and *Homarus*, he used only a single preserved specimen of each and their differences from *Carcinus* were as prominent as their similarities. Burkenroad (1947) proposed a general theory that attempted to incorporate the discrepancies he had observed: he suggested that some eggs required an "intensifier substance" for attachment, whereas others were "self-fusible," resulting in both egg-to-egg and egg-to-seta attachment. He neglected the possibility, however, that egg-to-egg attachment might result from the hardening of an encompassing cement, such as the pouch of glaire observed by Andrews (1906), rather than activity by individual eggs.

The variations observed in different species prohibit description of a general theory or a typical system for decapod egg attachment. It may be possible, however, that there is similarity in their need for egg support during attachment. The carideans, as exemplified here, may have a fast-acting, externally applied adhesive material that attaches eggs held in place by long pleopods while the female stands upright. The macruran adhesive material may act more slowly (Andrews, 1906; Yonge, 1937) and, coupled with relatively shorter pleopods, may necessitate the unique and highly vulnerable inverted posture maintained during egg attachment. Brachyurans may have slowly attaching eggs (Cheung, 1966), apparently without benefit of an externally applied adhesive material, but attachment may be aided by their anatomical enclosure for the eggs (flexed abdomen) and, for some crabs, by their action of burying themselves in sand to support the eggs. These differences might reflect variations in the reproductive strategies of each section (infra-Order) of the Decapoda.

ACKNOWLEDGMENTS

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FIGURE 18. A pleopod cross-section before ecdysis, shows the mucilage packets (mp) replete with dark-staining material. In this section, they are found close to the periphery of the pleopod, just inside the elongate epithelial cells (ec). Toluidine blue stain, 120×.

FIGURE 19. Cross-section of a pleopod immediately after ecdysis, shows the elongated epithelial cells are still present, but the mucilage packets are depleted (dmp) of their dark-staining material. The adhesive material (adh), which appears at this time on the external surfaces of the pleopod and pleopodal setae, is believed to be the dark-staining material secreted from the mucilage packets. Toluidine blue stain, 285×.

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EGGS OF *PALAEMON MACRODACTYLUS:* II. ASSOCIATION WITH AQUATIC BACTERIA

WILLIAM S. FISHER

Aquaculture Program, Animal Science Department, University of California, Davis, CA 95616

ABSTRACT

Eggs of the shrimp, *Palaemon macrodactylus,* were studied under various conditions to determine their susceptibility to colonization by aquatic bacteria. Under constant conditions in a laboratory system, bacterial populations monitored over the shrimp's two-week brooding period reached a maximum by seven days and stabilized. Nutrient addition increased bacterial populations substantially and retarded embryonic development, a probable result of hypoxia. Bacterial populations on eggs that had been detached from the egg mass and suspended in the system water increased by nearly an order of magnitude, as did populations on eggs brooded by females whose first pereiopods, or "cleaning chelipeds," had been excised. This result implied that preening activities of the first pereiopods were responsible for dramatically reducing bacterial populations. Bacterial populations on fertilized and unfertilized eggs responded alike to conditions of detachment and nutrient addition, indicating no apparent differences caused by mating, fertilization or development. Eggs extruded without benefit of attachment by the outer investment coat, a condition artificially produced by excising the pleopods, supported high bacterial populations. This may have resulted from additional nutrient escaping from the ooplasm or the greater susceptibility of the exposed vitelline envelope.

INTRODUCTION

Externally incubating crustacean embryos are exposed to the aquatic environment for extended durations. For example, the Pacific coast Dungeness crab, *Cancer magister,* broods its embryos for nearly 3 months, and the American lobster, *Homarus americanus,* has a 9 month incubation period. During this time, the embryos are a potential substrate for aquatic bacteria that are attracted to solid surfaces. Nutrient concentration at solid-liquid interfaces has been shown to attract bacteria (Marshall, 1976), and in low-nutrient (oligotrophic) environments, association with surfaces is frequently necessary for bacterial growth (Heukelekian and Heller, 1940; ZoBell, 1943). Several investigators (Henrici, 1933; Zobell and Allen, 1933; Marshall *et al.,* 1971; Corpe, 1973) have described the colonization and succession of microorganisms on surfaces of submerged glass slides. Within 5 days, complex microbial communities develop which include stalked and budding bacteria, diatoms, and protozoans.

In externally brooding decapods developing embryos are attached beneath the abdomen on the pleopods of the female. Water is circulated through the egg mass which acts like a filter by accumulating debris (Bauer, 1979). This build-up of organic debris and microorganisms is often detrimental to the host embryos. Fisher (1977) lists some reports of microbial epizoic diseases on decapods and suggests that microorganisms restrict gaseous exchange across the embryonic surface by both phys-

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ical means and respiratory competition, causing asphyxiation of the embryo. Working with hatchery-incubating eggs of salmon and trout, Trust (1972) found that bacteria covering only 1–10% of the available surface of an egg could consume oxygen at a rate five times faster than the egg. Oppenheimer (1955) and Bell (1966) have suggested that enzymes and toxins produced by surface microorganisms could damage embryos. Bell and co-workers (1971) however, studied the bacterial flora of stream-incubating Pacific salmon eggs and suggested bacterial fouling occurs only after eggs die. They suggested that heavy fouling on live eggs occurs only in culture, where conditions stimulate bacterial growth.

Presumably, healthy decapod embryos exposed for long periods of time have some mechanism(s) to restrict the growth of microorganisms on their surfaces. Possibilities include "preening" by the female (Bauer, 1979), maternal secretions such as the antibacterial agglutinating exudate of the aquatic arthropod, *Limulus polyphemus* (Stagner and Redmond, 1975), and embryonic secretions, such as the antimicrobial glucanase (Horikoshi *et al.*, 1963), which is released by sea urchin eggs during the fertilization reaction (Epel *et al.*, 1969).

This study examines eggs of an estuarine shrimp, *Palaemon macrodactylus*, in an attempt to elucidate some of the factors that determine the number of bacteria that inhabit the surfaces. The term "eggs" is often used in a general sense to mean unfertilized eggs, fertilized eggs, or both. Where specific designation is relevant, the terms "unfertilized eggs" and "fertilized eggs" or "embryos" will be used.

MATERIALS AND METHODS

The animals

Adult estuarine shrimp, *P. macrodactylus*, were collected from the mouth of the Petaluma River in northern California and transported to the University of California, Davis campus, where they were held in a closed recirculating system. They were placed in individual 8 × 15 cm compartments (10 cm water depth) in a 1.5 m × 30 cm plexiglass tank. A small jet of water was splashed into each compartment and was drawn through sand, pebbles, and oyster shell in an undergravel filter. Water temperature was maintained at 21–25°C; salinity fluctuated between 10–20 g/l; light:dark regime was 16:8 and animals were fed daily with live adult *Artemia salina* or frozen bay shrimp, *Crangon*. Mating of *P. macrodactylus* was most successful when a male was placed with a female a few days before her expected molt. If not mated, females extruded and attached unfertilized eggs, but they were usually removed or shed within 3 days.

Monitoring bacterial populations

Bacteria were enumerated by removing a cluster of attached eggs or embryos from the second pleopod of the brooding female and dissecting five connected eggs from the cluster as one sample. The samples were rinsed five times with sterile sea water diluted 1:2 (=1/3 SW) in a sterile porcelain dish, then homogenized in a sterile 5 ml Potter-Elvehjem tissue grinder (0.10–0.15 mm clearance). Using standard bacteriological procedures, the homogenized sample was diluted and plated in triplicate on a diluted concentration of Difco Marine Agar 2216 (=1/3 MA). Dilution was employed to simulate estuarine salinities; control plating showed that the dilution of nutrient in the media did not reduce bacterial counts. Bacterial colonies were counted after incubation for 3 days at 25°C. Egg clusters of 18 females were sampled directly from the Petaluma River on 16 June 1981 to determine bacterial levels in nature.

Bacterial populations from embryos and unfertilized eggs attached to various females in the system were monitored intermittently over a 3-month period. In an eight-day span during this period, system water from individual compartments was simultaneously monitored with embryo samples to determine whether bacterial fluctuations within the system or over time would alter the trends observed on the embryo samples. To accomplish this, 15 water and embryo samples were collected from 4–6 compartments on three separate occasions within the 8 days. Water samples were plated and incubated as previously described for embryo samples.

Effects of attachment

Unattached, unfertilized eggs were obtained by excising the first or second pair of female pleopods immediately prior to egg extrusion. As the eggs were extruded, they fell unattached to the floor of the tank where they clumped together. Within hours, the eggs were rinsed and placed, as a clump, in moving, well-aerated system water, and bacterial populations were monitored. Clusters of about 50 detached embryos, that is, embryos artificially removed from the second pleopods of the female, were clipped from various females, rinsed three times in sterile 1/3 SW, and suspended in system water by a sewing thread wrapped around the connectants between the embryos. These detached embryos were suspended either in uninhabited compartments of the system or in 125 ml flasks with 100 ml of system water that was changed daily. Sterile aeration was provided via 1 ml pipettes inserted through vented stoppers in each flask. In five separate experiments, embryos were detached 7, 7, 10, 11, and 12 days after extrusion, and bacterial populations were monitored for 1–7 days after detachment. Embryos remaining attached to the female were also monitored for comparison.

The first or third pair of pereiopods, which correspond respectively to the "cleaning chelipeds" and "first walking legs" described by Bauer (1979), were excised from two mated females who had extruded eggs on the same day. The pereiopods were excised 3 days after oviposition, and bacterial populations were monitored intermittently thereafter until hatching. In two other females, the first pereiopods were excised 1 day and 6 days after their eggs were oviposited, and bacterial populations were monitored until hatching.

Effects of fertilization

Nonmated females attached extruding eggs for only a few days before removing them. To determine the effect of fertilization on the bacterial population, these eggs were monitored during this short attached period for comparison with fertilized eggs during the same period. In addition, unfertilized and fertilized eggs of the same age (1-day-old) were detached and suspended in flasks containing system water or system water plus nutrient (10% Difco Nutrient Broth) and bacteria were monitored 2 days later. This experiment was repeated once in 5% yeast extract and once using 1-day-old unfertilized eggs and 7-day-old fertilized eggs in system water and system water plus 10% yeast extract.

Effect of environmental nutrient and bacteria

Clusters of 5-day-old embryos were detached and suspended in four separate flasks of system water with 0%, 1%, 10%, and 20% concentrations of nutrient, a 1:2

dilution of Difco Marine Broth 2216 (=1/3 MB). System water and nutrient were replenished daily, and bacterial numbers were monitored for 7 days.

Attached and detached embryos were compared in their response to nutrient addition by suspending detached embryos (7 and 11 days old) in a 600 ml closed, aerated compartment with the parent female and her remaining complement of attached embryos. System water was supplemented with a 2% concentration of 1/3 MB and was replenished daily. Bacteria were monitored from attached and detached embryos.

Clusters of 7-day-old embryos were excised and suspended in separate aerated flasks of system water and system water plus inorganic nutrients (50 mg/l $NaNO_3$ and 50 mg/l NaH_2PO_4). Bacteria were monitored for the next 7 days from the two flasks of detached embryos and from those remaining attached on the female in a system compartment. Water in the flasks was not replenished, and nutrient was added only once.

A bacteria isolated from embryos of *P. macrodactylus* was incubated for 19 hours at 25°C in a 1/3 SW-yeast extract broth, centrifuged into a pellet, and re-suspended in sterile 1/3 SW at a concentration of 6×10^{10} cells/ml. Clusters of about 50 detached 1-day-old embryos were placed in test tubes containing 9 ml of this inoculum, dilutions of this inoculum, and sterile 1/3 SW. Embryo samples were taken at 2 hours and 24 hours to monitor bacterial populations.

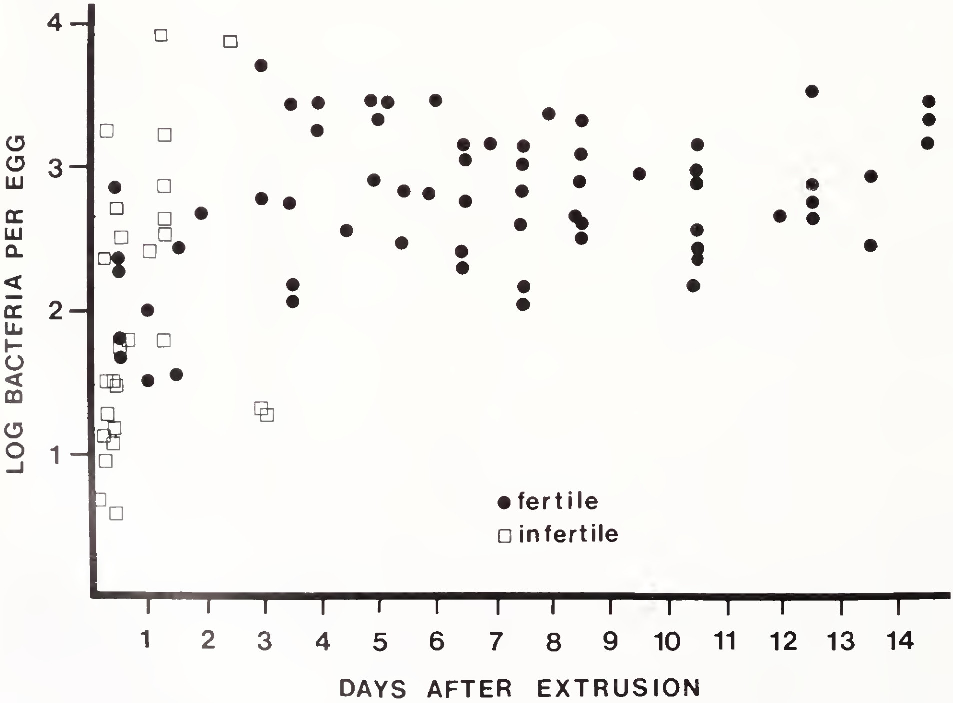


FIGURE 1. Numbers of bacteria on eggs attached to females held in the laboratory system. The period of attachment was 14 days for fertilized eggs and 1–3 days for unfertilized eggs. Each point on the graph represents the average count from a sample of five eggs detached from the second maternal pleopod, homogenized, then plated in triplicate on 1/3 MA.

TABLE I

Comparison of bacterial populations on embryos detached from the female pleopods with those remaining attached to the pleopods

		Number of bacteria/embryo	
Experiment	Age (days)	Attached	Detached
1.	$t_0 = 7$	1.5×10^2	
	8	4.6×10^2	7.1×10^2
	9	9.3×10^2	3.3×10^3
	10	8.1×10^2	4.0×10^3
2.	$t_0 = 7$	1.0×10^3	
	8	4.2×10^2	4.6×10^3
	10	1.4×10^3	7.2×10^3
	12	3.4×10^3	5.1×10^3
	14	2.1×10^3	6.8×10^3
3.	$t_0 = 10$	6.9×10^2	
	11	5.2×10^2	4.1×10^3
4.	$t_0 = 11$	8.3×10^2	
	12	1.4×10^3	3.9×10^3
	13	1.4×10^3	2.4×10^4
5.	$t_0 = 12$	8.7×10^2	
	13	1.1×10^3	9.8×10^3

t_0 = embryonic age at time of detachment.

RESULTS

The animals

Female *P. macrodactylus* held in the system molted every 16.3 (±3.5) days and eggs were extruded and attached to the pleopods after 76.5% of these molts, usually within 1 day. Fertilized eggs incubated an average 14.3 (±0.8) days, whereas unfertilized eggs remained attached only 1–3 days before being removed by the female.

Monitoring bacterial populations

Natural levels of bacteria determined from 18 females collected from nature averaged 118 bacteria/embryo (range = 10–600). Embryos attached to females held in the system showed an initial increase in bacterial numbers that leveled by the second week of brooding (Fig. 1). Samples monitored during the second week of brooding fluctuated near 10^3 bacteria/embryo. Populations in the system water over an 8-day period averaged $1.4 (\pm.64) \times 10^5$, $3.2 (\pm.88) \times 10^4$, and $1.6 (\pm.93) \times 10^5$ bacteria/ml on three different sampling days. Plotting the ratios of bacteria per embryo to bacteria per milliliter system water showed no differences in the relative positions of each sample or the shape of the curve depicted in Figure 1. Unfertilized eggs showed increasing bacterial populations during the few days they were attached.

Effects of attachment

Unattached eggs (those extruded from females with excised first or second pleopods) had high bacterial populations of 1.7×10^5 bacteria/egg within 1 day and 2.7

TABLE II

Bacterial populations on embryos attached to females with excised first pereiopods, the "cleaning chelipeds"

Experiment	Age (days)	Number of bacteria/embryo
1.	$t_0 = 3$	2.8×10^2
	5	4.3×10^3
	7	3.3×10^3
	11	1.4×10^4
2.	$t_0 = 6$	5.5×10^3
	7	4.9×10^4
	10	1.5×10^4
	12	5.9×10^4
3.	$t_0 = 1$	7.4×10^1
	3	7.4×10^2
	5	4.9×10^3
	9	3.2×10^4
	10	7.1×10^4

t_0 = embryonic age at time of excision. Bacterial numbers for t_0 days were obtained prior to excision.

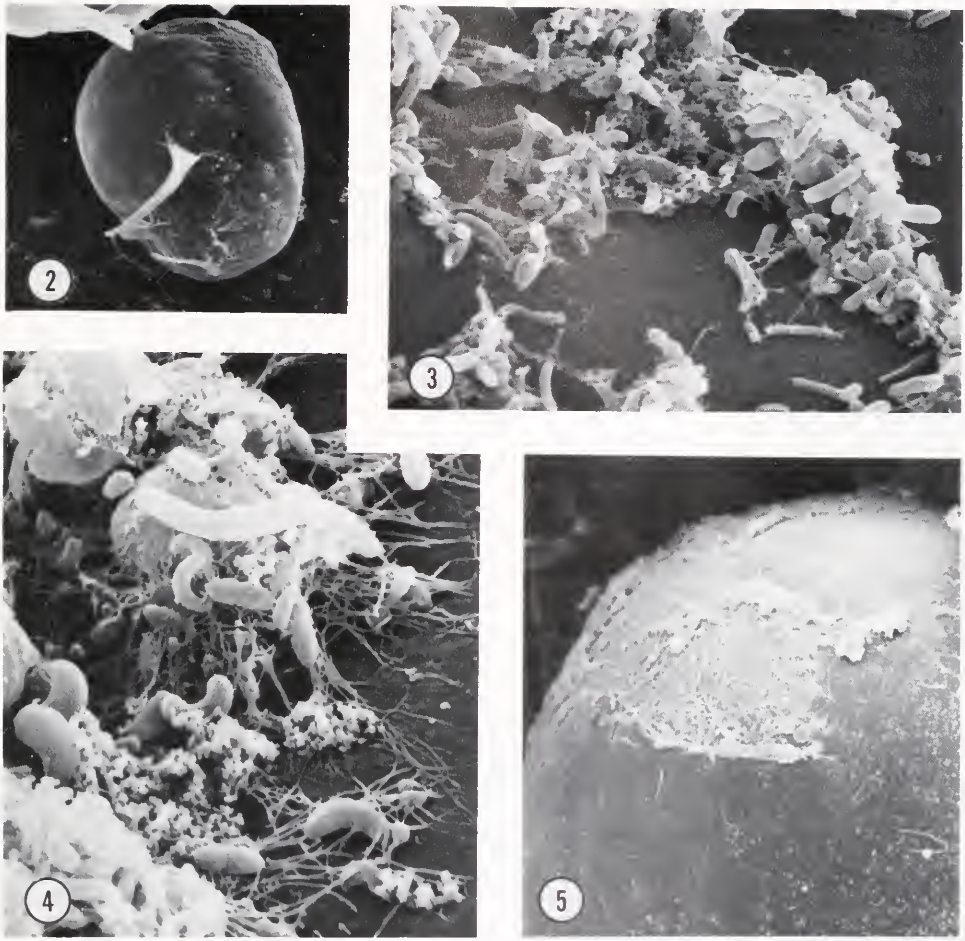
$\times 10^5$ bacteria/egg within 2 days after extrusion. During this time, the green yolk turned white, dissipated, and eventually only the translucent vitelline envelope remained. These unattached eggs did not have an outer investment coat (Fisher and Clark, 1983). Bacterial populations on detached embryos were higher than those remaining attached to the female pleopods (Table I). Numbers of bacteria on attached embryos were an average 72% less than those found on detached embryos, and those on embryos from the female with her walking legs excised were an average 73.8% less than those from the female with her cleaning chelipeds excised. Bacterial populations increased on all females with excised chelipeds (Table II), and scanning electron micrographs (Figs. 2–5) showed egg surfaces from normal females (with intact cleaning chelipeds) to have patchy distributions of bacteria.

Effects of fertilization

Bacterial populations on attached eggs monitored over the first three days showed large variation and no distinguishing differences between fertilized and unfertilized eggs (Fig. 1). Eggs and embryos detached within 1 day of extrusion and suspended in system water or system water plus nutrient, had equivalent bacterial populations after 3 days (Table III). Likewise, populations on unfertilized eggs detached within 1 day responded in a manner nearly identical to populations of fertilized eggs detached after 7 days of brooding.

Effect of environmental nutrient and bacteria

Incremental concentrations of nutrient (1/3 MB) increased populations of bacteria on detached 5-day-old embryos (Fig. 6). Populations on control embryos (0%) increased one order of magnitude before leveling at 10^4 bacteria/embryo. Daily addition of 1% nutrient caused an initial increase of nearly two orders of magnitude that also stabilized. Bacteria on embryos held in 10% nutrient exceeded 6×10^5 bacteria/embryo in 2 days, but measurements were discontinued because of a fungal



FIGURES 2–5. Scanning electron micrographs of bacteria on the surfaces of embryos from normal females (cleaning chelipeds intact). In Figure 2 (80×), only a small portion of the embryo is inhabited by bacteria, but these bacteria appear capable of forming colonies as shown in Figure 3 (5,000×). The most heavily infested areas show a variety of microbial epizoots such as those shown in Figure 4 (7,500×). Patchiness may be due to preening or scraping by the female's cleaning appendages, which could account for the rolled mats of microorganisms shown in Figure 5 (240×).

infection. Bacterial populations on embryos suspended in 20% nutrient increased to 10^6 bacteria/embryo within 2 days and 10^8 bacteria/embryo within 7 days. Twelve-day-old embryos in 20% nutrient were approximately 2–3 days retarded in their development, as indicated by eyespot size and appearance, whereas embryos in the 0% and 1% nutrient developed within the normal time period.

Both attached and detached embryos monitored from the time of detachment, showed increased bacterial populations when held in a 2% concentration of 1/3 MB (Fig. 7), but bacterial counts on attached embryos were less (by an average 85.2%) than those on detached embryos. Hatching success and larval survival was poor from the detached embryos whereas hatching appeared to occur normally from the attached embryos and larval survival was excellent.

TABLE III

Comparison of bacterial populations on fertilized and unfertilized eggs detached from maternal pleopods and suspended in the same flask containing either system water or system water with a nutrient additive

	Unfertilized		Fertilized	
Experiment	Age (d)	Number of bacteria per egg	Age (d)	Number of bacteria per egg
1. No nutrient	1	1.7×10^3	1	7.2×10^1
	3	4.3×10^4	3	4.0×10^4
10% Nutrient Broth	1	1.7×10^3	1	7.2×10^1
	3	2.1×10^5	3	1.2×10^5
2. 5% Yeast Extract	1	5.1×10^2	1	6.5×10^2
	3	1.5×10^5	3	1.1×10^5
3. No nutrient	1	1.3×10^1	7	1.1×10^2
	2	3.2×10^3	8	5.6×10^3
	3	8.3×10^3	9	5.0×10^3
10% Yeast Extract	1	1.3×10^1	7	1.1×10^2
	2	3.2×10^4	8	1.9×10^4
	3	8.3×10^4	9	6.2×10^4

Seven-day-old embryos suspended in system water with a single addition of inorganic nutrients showed an increase in bacteria of two orders of magnitude after 1 day (Fig. 8); this number gradually declined during the experimental period and resulted in successful hatching of larvae. Embryos suspended in unreplenished system water without nutrient addition increased to 4.6×10^3 bacteria/embryo after 1 day then leveled until hatching. Those embryos remaining attached to the female maintained an average population of 10^3 bacteria/embryo throughout the experiment.

One-day-old embryos held in water of various concentrations of bacteria showed an early response to the higher concentrations, but such differences diminished after only 24 hours (Table IV). The highest counts on the embryos, 7.4×10^5 bacteria/embryo, were observed after 2 hours exposure to the highest inoculum (6×10^{10} bacteria/ml). By 24 hours, the bacterial count had decreased to 3.9×10^4 bacteria/embryo, while the bacterial count from the control tube increased from 6.3×10^2 (2 hours) to 6.0×10^3 bacteria/embryo by 24 hours. Bacterial numbers from this experiment cannot be compared with numbers from other experiments in this study since experimental conditions were different.

DISCUSSION

Bacterial populations on brooding embryos of the estuarine shrimp, *P. macrodactylus,* were restricted to approximately 10^3 bacteria/embryo during the major portion of the shrimp's 14-day incubation period in laboratory conditions. Increasing environmental nutrient increased the bacterial population (Fig. 6), and continuous addition of high levels of nutrient retarded the development of embryos. Respiration of bacteria and their physical presence on an embryo surface reduces the amount of oxygen reaching the embryo and, if sufficient numbers of bacteria are present,

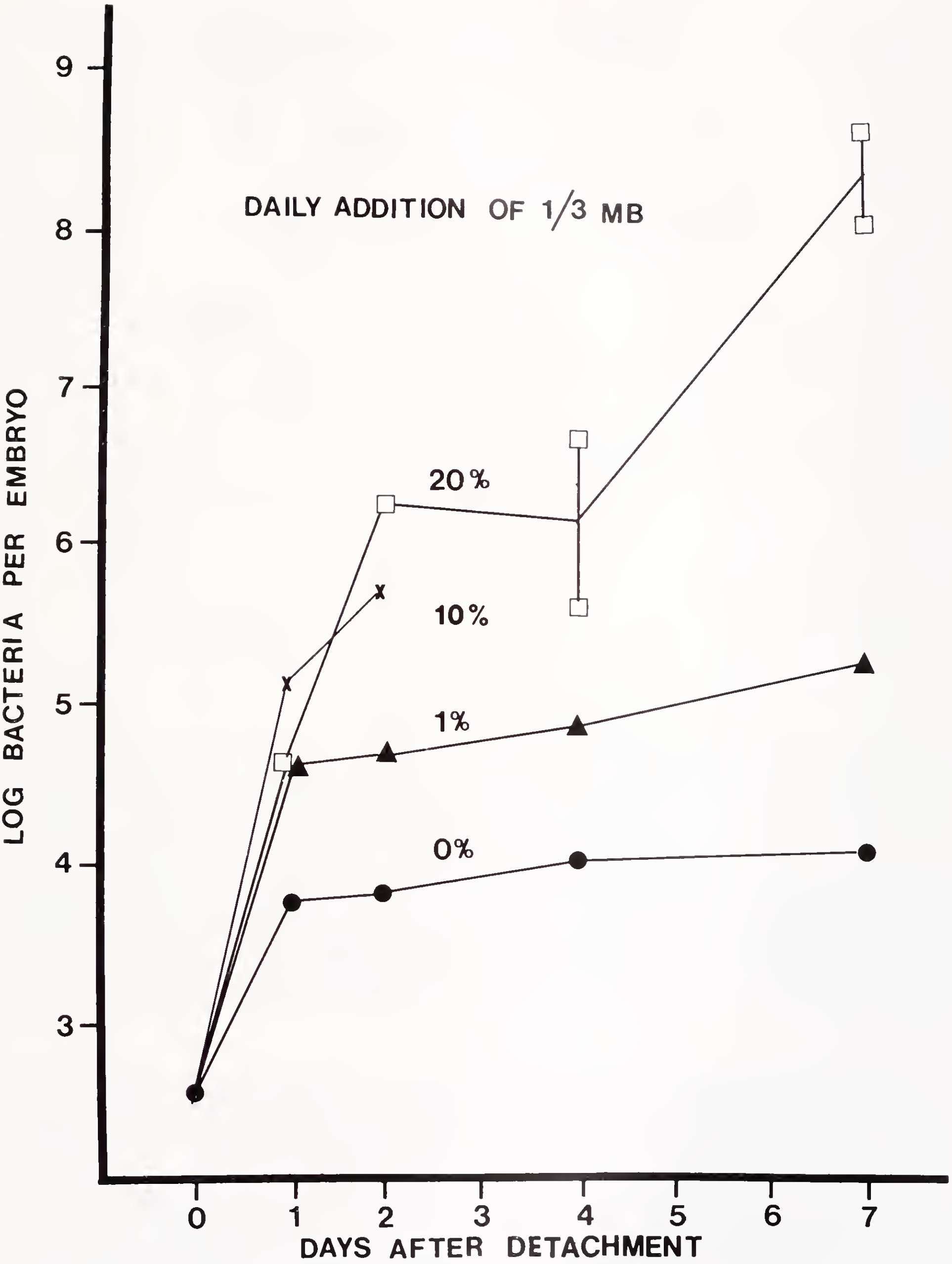


FIGURE 6. Numbers of bacteria on five-day-old embryos detached from a single female and suspended in different concentrations of nutrient (1/3 MB) that was added daily with the water change. Embryos held in 10% nutrient were infected by fungus two days after detachment and eventually died. Those held in 20% nutrient were 2–3 days retarded in development by the seventh day after detachment.

may create a respiratory stress condition for the embryo. Oxygen requirements of *P. macrodactylus* embryos increase dramatically at about 9 days (unpublished data) which may be a particularly vulnerable period.

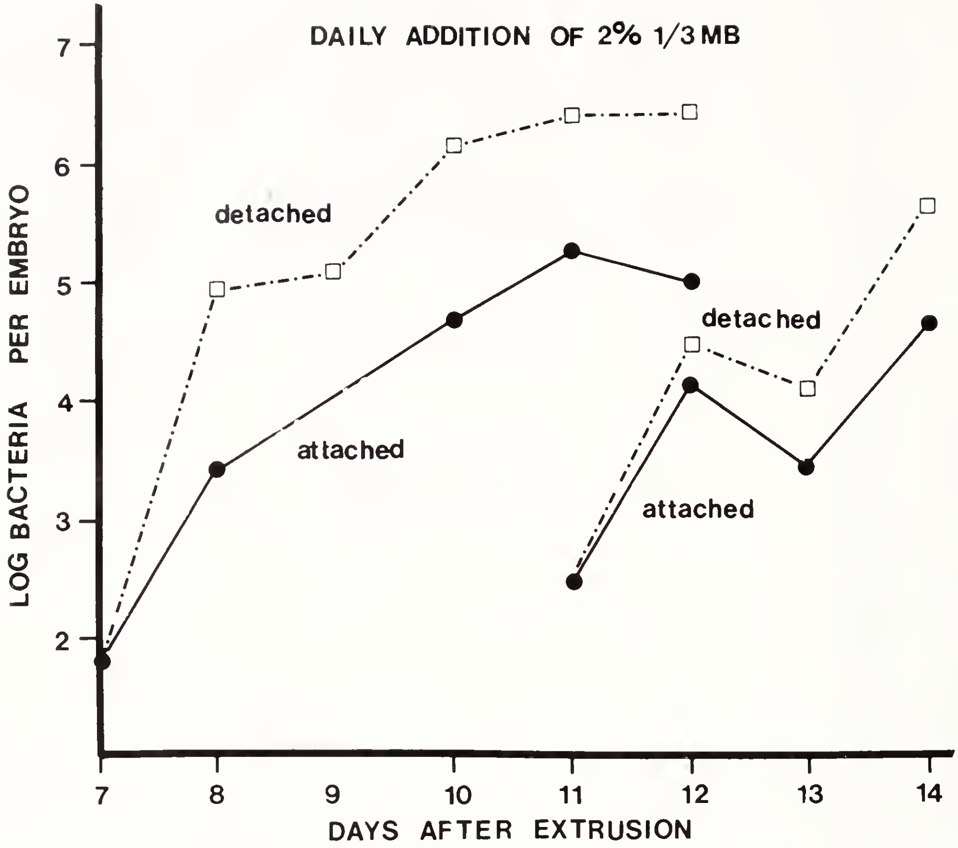


FIGURE 7. Numbers of bacteria on embryos detached from two females, one after seven days of brooding and the other after eleven days of brooding, and numbers of bacteria on embryos remaining attached to those females. Detached embryos were suspended in the same compartments with the respective donor female and the remaining complement of attached embryos. Nutrient (1/3 MB) was added at a 2% concentration with the daily water change. Hatching of the older embryos was successful for those remaining attached on the female, but detached embryos had poor hatching success and poor larval survival.

Another detrimental condition may result from bacteria inhabiting embryo surfaces. Bacteria isolated from the surfaces of *P. macrodactylus* embryos (Fisher, 1983) produced extracellular enzymes capable of degrading (among other substrates) chitin and lipid, two structural components of decapod cuticles and egg coats (Yonge, 1937). Bell (1966) suggested that bacterial release of degrading enzymes caused damage to fish egg coats and ultimately to the embryo. Such a phenomenon might also be expected in the decapods, since it has been well established that chitinolytic bacteria (Hess, 1937; Rosen, 1967) and possibly lipolytic bacteria (Baross *et al.*, 1978) are capable of penetrating the tough decapod exoskeleton to create shell diseases.

Eggs that were extruded without benefit of attachment (obtained by excising the first or second maternal pleopods before extrusion) lacked an outer investment coat provided by the female pleopods (Fisher and Clark, 1983). These unattached eggs rapidly deteriorated and, within 2 days, only the vitelline envelope remained ap-

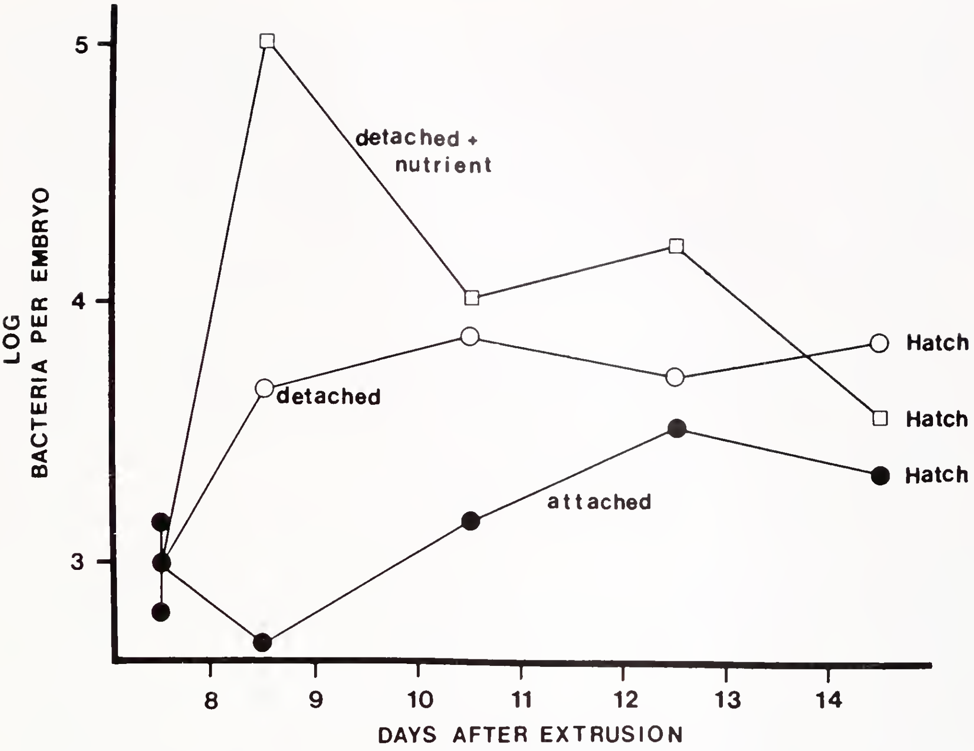


FIGURE 8. Numbers of bacteria on seven-day-old embryos detached from a single female and suspended in system water (detached) or system water plus a single addition of the inorganic nutrients $NaNO_3$ and NaH_2PO_4 at 50 mg/l each (detached + nutrient), and on embryos remaining attached to the female which were held in a system compartment (attached). Water was not replenished during the experiment. Successful hatching occurred from all treatments.

pearing as a translucent "ghost capsule." During this time, a high number of bacteria ($1.7–2.7 \times 10^5$ per egg) inhabited the surfaces, implying that a high and constant level of nutrient was available from the vitelline envelope itself or from leakage of ooplasm through the vitelline envelope.

TABLE IV

Bacterial populations found on one-day-old embryos detached from the same female and held in tubes of increasing concentrations of bacteria[1]

Initial inoculum (bacteria/ml)	Number of bacteria/embryo[2]	
	After 2 hours	After 24 hours
Control (=0)	6.3×10^2	6.0×10^3
6.0×10^4	1.1×10^3	7.8×10^4
6.0×10^6	1.2×10^3	1.1×10^4
6.0×10^8	7.9×10^3	4.2×10^4
6.0×10^{10}	7.3×10^5	3.9×10^4

[1] Bacteria for inocula were isolated from an embryo surface and cultured from a single colony.

[2] Numbers of bacteria were monitored from the same tubes held at room temperature 2 hours and 24 hours after inoculation.

Embryos that were detached from the brooding pleopods consistently had more bacteria than embryos remaining attached. A comparably high number of bacteria were found on embryos of females whose first pereiopods (cleaning chelipeds) had been excised, indicating that preening behavior may be responsible for controlling a major portion of the bacterial population. Scanning electron micrographs (Figs. 2–5) support this hypothesis by showing patchy distributions of bacteria, possibly corresponding to areas raked by cleaning setae of the first pereipods (Bauer, 1979). Infection of eggs by a fungus, *Lagenidium callinectes*, has also been shown to increase when the cleaning chelipeds are excised (Fisher, 1983).

Fertilization had no apparent effect on the bacterial numbers, since populations monitored on unfertilized eggs were the same as those on fertilized eggs under conditions of attachment, detachment, and nutrient addition. The initial number of bacteria in the environment was a relevant factor for only one day, after which populations on embryos held in high bacterial concentrations receded to levels found on embryos held in low bacterial concentrations. Similarly, populations on embryos treated with only a single addition of nutrient increased temporarily but eventually declined to levels found on untreated embryos.

Figure 8 depicts a single experiment that aids in summarizing some of the results of this study. Bacterial populations on detached embryos fluctuated around 10^4 bacteria/embryo while the populations on attached embryos were reduced by female preening activities to about 10^3 bacteria/embryo. By analogy, it can be estimated that nutrient in the Petaluma River (where natural samples were obtained) was capable of supporting 10^3 bacteria/embryo and was reduced by female preening to the measured 10^2 bacteria/embryo. A single addition of inorganic nutrient to the test water of detached embryos allowed the bacterial population to increase initially (Fig. 8), but numbers subsequently dropped to near the control level. This probably reflects competition between bacteria for space or nutrient. When nutrient is continuously added, bacterial populations are maintained at increased levels (Fig. 6). In this context, it is also interesting that high bacterial populations in water without additional nutrient did not remain associated with the embryos (Table IV). Further study may reveal a relationship between nutrient levels and bacterial attachment to embryos.

It has been shown here that *P. macrodactylus* embryos depend largely on low nutrient levels and maternal preening to restrict surface bacterial populations. The effect of nutrient on microbial populations inhabiting embryo surfaces has also been shown for the Dungeness crab, *Cancer magister* (Fisher, 1976). Since female crabs do not have the same preening ability as *P. macrodactylus* and carry roughly a million eggs, it is interesting to speculate whether crabs must depend on low nutrient levels or if there is some other form of defense.

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EGGS OF *PALAEMON MACRODACTYLUS:* III. INFECTION BY THE FUNGUS, *LAGENIDIUM CALLINECTES*

WILLIAM S. FISHER

Aquaculture Program, Animal Science Department, University of California, Davis, CA 95616

ABSTRACT

Eggs of the estuarine shrimp, *Palaemon macrodactylus,* were studied under various conditions to determine their susceptibility to a known fungal pathogen, *Lagenidium callinectes.* Eggs exhibited no signs of fungal infection under normal conditions, but were infected when detached from the female or when the female's first pereiopods ("cleaning chelipeds") were excised. This implied that preening or egg removal by the first pereiopods halted infection or obviated detection of the fungus. Nutrient addition increased the number of infections and decreased the time to infection. Fertilized eggs were less susceptible to infection than unfertilized, probably the result of the hardened fertilization membrane restricting fungal penetration. Similarly, older embryos with progressively hardened coats were also less susceptible. Defense may have also been provided by antifungal bacteria isolated from embryo surfaces. Two of these epizooic bacteria belong to the marine genus, *Alteromonas,* and release extracellular enzymes, including lipase and chitinase. Both bacteria were found to inhibit a variety of pathogenic fungi, including six strains of *Lagenidium callinectes,* two strains of *Halipthoros milfordensis* and a freshwater *Saprolegnia* species.

INTRODUCTION

Two phycomycetous fungi have repeatedly been found to infect crustaceans in nature and in aquaculture situations. Couch (1942) first described *Lagenidium callinectes* parasitizing eggs of the blue crab, *Callinectes sapidus.* The fungus has subsequently been found pathogenic to penaeid shrimp larvae (Cook, 1971; Lightner and Fontaine, 1973), cultured larvae of the American lobster, *Homarus americanus* (Nilson *et al.,* 1976), and in zoeae of the Dungeness crab, *Cancer magister* (Armstrong *et al.,* 1976). A complete description of this phycomycete isolated from blue crab eggs has been provided by Bland and Amerson (1973). The pathogenic effects of another fungus, *Haliphthoros milfordensis,* were described (Fisher *et al.,* 1975) on *Homarus americanus* and *Homarus gammarus* postlarvae in aquaculture systems. *H. milfordensis* was later found to infect adult penaeid shrimp, ova and larvae of the brine shrimp, *Artemia salina,* and eggs of *C. sapidus* (Tharp and Bland, 1977). An unidentified fungus was also reported to infect detached eggs of *Palaemon idae* (Aiyer, 1949).

Chemical control of these destructive pathogens in aquaculture systems has been investigated by a variety of authors (Delves-Broughton, 1974; Armstrong *et al.,* 1976; Bland *et al.,* 1976; Fisher *et al.,* 1976; Abrahams and Brown, 1977). Antibiotic control has not been investigated and, historically, there have been very few references to microbial activity against fungi (Horikoshi and Iida, 1958). There are, however, several marine bacteria known to produce substances toxic to other bac-

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teria. Four species of the genus *Alteromonas* produce antibiotics against Gram-positive and some Gram-negative bacteria: *A. rubra* (Gauthier, 1976; Ballester *et al.*, 1977), *A. luteoviolaceus* (Anderson *et al.*, 1974; Gauthier and Flatau, 1976), *A. citrea* (Gauthier, 1977), and *A. aurantia* (Gauthier and Breittmayer, 1979). Several strains of other *Alteromonas* species (*A. haloplanktis*, *A. undina*, and *A. espejiana*) have been found off the coast of northern California (Chan *et al.*, 1978), but none were tested for antibiotic activity. Burkholder *et al.* (1966) describe a bacterium, *Pseudomonas bromoutilis* strain 287, that matches *A. haloplanktis* in all the recorded metabolic characteristics and does produce an antibiotic substance. None of these have been tested against fungi.

Palaemon macrodactylus, an estuarine shrimp introduced to the San Francisco Bay area from the Orient around 1954 (Newman, 1963), has not been tested for susceptibility to fungal infections. This study describes the infection of laboratory-held adults and brooding embryos of the shrimp *P. macrodactylus* by the phycomycete *Lagenidium callinectes*. It also examines susceptibility and potential mechanisms of protection against infection, including the release of an antifungal substance by bacteria (*Alteromonas*) isolated from embryonic surfaces. The term "egg" is used in a general sense to mean fertilized egg, unfertilized egg, or both. Where appropriate, specific designations will be "unfertilized egg," and "fertilized egg" or "embryo."

MATERIALS AND METHODS

Egg susceptibility to fungal infection

Adult shrimp, *P. macrodactylus*, were held in a closed recirculating system and maintained as described previously (Fisher, 1983). Infective fungal propagules for inoculation experiments were obtained in the following manner: the fungus, isolated from previously infected eggs and designated L-Pm, was cultured on dilute Difco Marine Agar 2216 (1/3 MA). An agar slab with bacteria-free fungal hyphae was then transferred to dilute Difco Marine Broth (1/3 MB). After 2–3 days in 1/3 MB, hyphae grew out from the agar slab, and vigorous shaking for 10–15 seconds broke off fragments that could initiate new growth. Concentrations of these infective propagules were determined by plating 0.1 ml broth on 1/3 MA. Eggs infected with the fungus were fixed in 2% glutaraldehyde in a standard 0.1 M phosphate buffer at pH 7.6 and embedded in an epoxy medium (Spurr, 1969). Thick sections (0.5 μm) were stained with borate buffered toluidine blue (Dewel and Clark, 1972).

Two clusters of externally brooding 1-day-old embryos were detached from an ovigerous female and rinsed in sterile dilute sea water (sterile 1/3 SW). The clusters were suspended by thread into aerated 125 ml Erlenmeyer flasks containing 100 ml sterile 1/3 SW. One flask was inoculated with a 1 ml fungal suspension containing approximately 210 propagules and the embryos of both clusters were monitored for signs of infection for 7 days.

The first or third pair of pereiopods were excised from two mated females extruding eggs on the same day. Excision occurred 3 days after oviposition and embryos were examined intermittently for fungal infection until hatching. The first and third pereiopods correspond respectively to the "cleaning chelipeds" and "first walking legs" described by Bauer (1979). In similar experiments, the first pereiopods were excised from two other mated females, 1 day and 6 days after oviposition, and one unmated female whose first pereiopods were excised 1 day after extrusion of unfertilized eggs.

A series of tests were conducted to determine the effect of fertilization on egg susceptibility to L-Pm. In six separate experiments, 1-day-old unfertilized egg clusters were suspended in the same aerated flask with 1-day-old fertilized egg clusters and a fungal inoculum. To determine the effect of brooding time, embryos aged 2, 4, 7, 10 and 12 days were suspended and compared in three separate trials. Fungal inocula for these experiments ranged from 30–200 infective propagules and, in some cases, 1/3 MB was added as nutrient at a concentration of 5%.

Characteristics of the fungus, L-Pm

Samples of L-Pm were sent to Dr. C. Bland, East Carolina University, Greenville, North Carolina for examination. L-Pm was also plated by agar slab transfer to different agar media where radial growth rates were compared to six isolates of *Lagenidium callinectes* and two isolates of *Haliphthoros milfordensis*, all obtained through the courtesy of C. Bland and L. Crisp (East Carolina University), and one *Saprolegnia sp.* isolated from the white sturgeon, *Acipenser transmontanus*. Designations for these isolates are reported in Table II. The media used for fungal growth tests were 1/3 MA, shrimp infusion agar (SIA), and corn meal extract agar (CMA). SIA was prepared by autoclaving 10–15 medium-sized shrimp (*Crangon*) in 1 liter of 1/3 SW, straining, and adding 0.2% glucose. CMA was prepared by boiling 25 g of Albers yellow corn meal for 3 minutes in distilled water, filtering through 2 layers of cheesecloth, then adding distilled water to 1 liter. For 1/3 SW-CMA and 1/2 SW-CMA, appropriate dilutions of sea water were substituted for the distilled water. NaCl and KCl were added to CMA media at concentrations of 1% and 2.5% to determine gross salt and ion optima. In one medium, 1/2 SW-CMA was supplemented with 0.3% glucose (1/2 SW-CMAG). All media contained 2% agar. Agar slabs of the various fungi were plated on these media and growth rates measured over periods of 2–25 days.

Antagonistic effects of bacteria

Embryos from 15 *P. macrodactylus* females collected from the Petaluma River on 16 June 1981 were sampled for bacteria on 1/3 MA (Fisher, 1983), and 177 bacterial colonies, the total number from duplicate plates at the 10^{-2} dilution level, were re-cultured on the peripheries of 1/3 MA plates. Agar slabs of growing fungus (L-Pm) were placed in the center of the plates, and inhibition of growth was monitored as hyphae approached the individual bacterial colonies.

Two bacterial types, both isolated from embryos of females held in the laboratory system, maintained a large zone of inhibition when plated with L-Pm. One isolate, designated I-2, had been isolated from untreated control eggs in an unpublished experiment where eggs treated with penicillin had become infected with fungus. The other isolate, designated I-13, was isolated from eggs during routine monitoring. Inocula of I-2 and I-13 were added to a test tube containing 1/3 MB and an agar slab of growing L-Pm. Control tubes received the fungal slab only and after 1–2 days, fungal growth in the tubes was compared.

Three fungal slabs were transferred to each of two, 0.45 μm-pore size, membranes laying on dehydrated nutrient pads in sterile petri dishes (Nalgene Nutrient Pad Kit). The nutrient pad in one dish was hydrated with sterile 1/3 SW while the other was hydrated with an inoculum of bacterial isolate I-2. The bacteria were separated from the fungal slab by the membrane. For three successive days, one fungal slab was removed from each membrane and transferred to a 1/3 MA plate where growth was monitored. Fungus growing from the edge of the agar slab was trimmed away prior to transfer and tests were made to insure against possible bacterial leakage across the 0.45 μm membrane.

The effect of the antifungal bacteria on L-Pm was observed on whole-mount light microscopy, thick sections, and by scanning electron microscopy.

Characteristics of the bacteria

Bacterial isolates I-2 and I-13 were tested for Na^+ requirement, fermentation of glucose, sucrose, and fructose, production of the extracellular enzymes alginase, amylase, chitinase, gelatinase and lipase, and utilization of carbon sources according to the methods of Baumann and Baumann (1981). Flagella stains followed the method of Mayfield and Inniss (1977).

A variety of fungi were tested against antifungal bacteria by plating fungal slabs on agar and allowing time for growth to become well established, then streaking the bacteria 10–20 mm away from the growing hyphae. Inhibition was determined by comparing hyphae distance from the bacteria. The fungi involved and agar media used were previously described. The activity of various *Alteromonas* species were tested in a similar manner against the fungi L-F2, L-Pm, and S-At, all on 1/3 MA. The bacteria, obtained through the courtesy of Drs. P. Baumann and L. Baumann (University of California, Davis), included *A. macleodii* strains 113 and 107 (Type strain, ATCC 27126), *A. haloplanktis* strains 121 and 215 (Type strain, ATCC 14393), *A. espejiana* strain 261 (Type strain, ATCC 29659), *A. undina* strain 272 (Type strain, ATCC 29660), *A. communis* strain 8 (Type strain, ATCC 27118) and *A. vaga* strain 40 (Type strain, ATCC 27119).

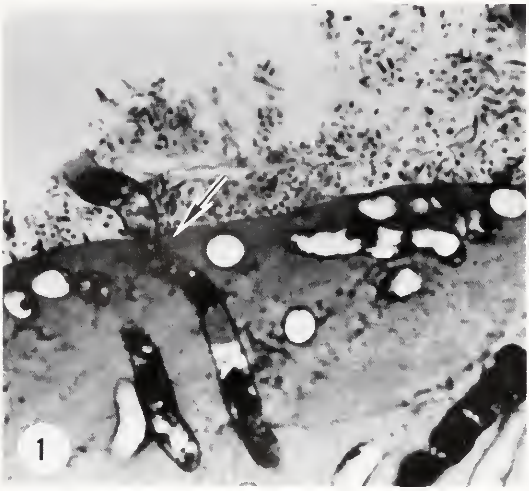
RESULTS

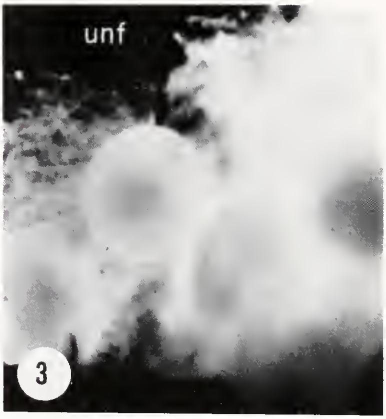
Egg susceptibility to fungal infection

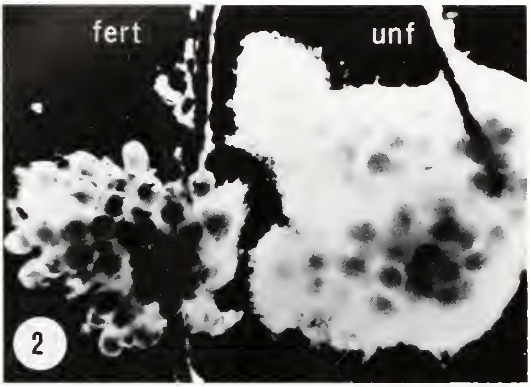
In all cases, embryos infected by *L. callinectes* neither survived nor hatched. Embryos suspended in a flask with fungal propagules were infected by the fungus on the third day following inoculation. The control embryos, detached from the same female but suspended in a flask without fungal propagules, showed no signs of infection after 7 days of observation. Fungus infecting unfertilized eggs penetrated the egg investment coats and totally invaded the internal milieu, destroying the otherwise apparent globular yolk structure (Fig. 1). Penetration of the egg coats appeared to cause a constriction of the fungal hyphae.

Embryos brooding on females with excised first pereiopods (cleaning chelipeds) became infected and died in all trials (Table I) whereas no dead or infected embryos were observed in the clutch of the female with her third pereiopods (walking legs) excised. No infected or dead eggs were observed in any other attached clutches throughout these studies. Excision of the first pereiopod of an unmated female (Table I, Experiment 4) allowed unfertilized eggs to remain attached for longer than 3 days, but over 80% of these eggs were infected by 5 days. All dead eggs and embryos plated on 1/3 MA showed growth of a fungus with the characteristics of L-Pm.

In all cases tested, unfertilized egg clusters were more susceptible to fungal infection than fertilized (Fig. 2). They were first to become infected and infection of all the eggs in the cluster occurred within 1 day of its first appearance on a single egg. Infection of fertilized eggs appeared 1–2 days after infection of unfertilized eggs and progressed more slowly through the cluster, requiring 3 days or longer to infect all the embryos. Age of the embryo did not consistently alter its susceptibility to the fungus, however the characteristics of the disease on mature embryos resembled







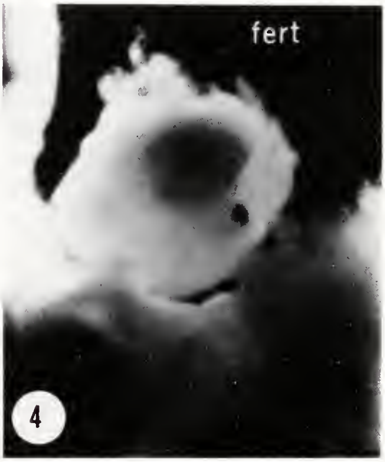


FIGURE 1. *L. callinectes* is constricted as it penetrates the outer egg layers (arrow) of this unfertilized egg. Fungal hyphae invade the internal milieu and destroy the otherwise globular ooplasm. Toluidine blue stain, 1,700×.

FIGURE 2. Unfertilized eggs were engulfed by fungal hyphae after three days incubation with L-Pm propagules while fertilized eggs suspended in the same flask were less susceptible. Even after five days, half the fertilized eggs remained uninfected.

FIGURES 3 and 4. The fungal hyphae on unfertilized eggs protruded directly outward from the egg surface whereas hyphae on fertilized eggs appeared to form a mat around the egg surface and the embryos may have been slowly asphyxiated rather than parasitized.

infestation rather than infection. Instead of penetrating each embryo individually (where hyphae could be seen protruding from the embryo surface as in Figure 3), a hyphal mat grew around the cluster and appeared to wrap the embryos (Fig. 4). First appearance of the fungus varied from 1–3 days for unfertilized eggs. Inoculated samples usually required 3 days for first appearance unless nutrient was added, which reduced the time to infection for both eggs and embryos.

Characteristics of the fungus, L-Pm

The fungus L-Pm was determined to be *Lagenidiaceous* by virtue of its extensively branched, sparingly septate hyphae of 6–10 μm cross-sectional diameter. Its

TABLE I

Fungal infection of attached fertilized and unfertilized eggs after excision of the female's first pereiopods, the "cleaning chelipeds"

Experiment number	Age (d)	Sample size (n)	% Infected
1. Fertilized	t_0* = 3	—	—
	5	25	3.8
	7	27	25.9
2. Fertilized	t_0 = 6	—	—
	10	24	4.0
	12	80	37.5
3. Fertilized	t_0 = 1	—	—
	5	30	0.0
	10	75	33.0
	12	95	84.2
4. Unfertilized	t_0 = 1	—	—
	5	17	82.4

* Embryonic age at time of excision is designated t_0.

No fungal infection was ever encountered on eggs attached to females with intact cleaning chelipeds. All infections were fatal.

radial growth rate on agar plates of different media and salt concentrations is shown in Table II. It compares most favorably with *L. callinectes* isolate L-F2, which was previously isolated from northern California waters. Both show rapid growth at 1%

TABLE II

Growth rates of various fungal isolates on agar plates of different media, measured in millimeters of radial growth per day

Media	L-1	L-6	L-3b	L-815	CE	L-F2	L-Pm	H-2	H-222	S-At
1/3 MA	0.5	1.0	2.0	1.2	2.4	10.0	11.9	0	0	18.5
SIA	0.3	0.9	1.7	0.5	2.7	9.7	6.5	*	0.7	15.0
CMA		*	1.7	0.7	0.9	6.8	7.2	0	0	17.0
1% KCl-CMA		0	1.0	0.5	0.9	7.2	7.3	*	0	12.5
2.5% KCl-CMA		0	0.5	0.6	0.4	4.0	4.2	0	0	2.5
1% NaCl-CMA		0.7	2.3	1.6	2.3	9.5	9.2	0.7	1.3	12.0
2.5% NaCl-CMA		0.7	0.8	0.5	1.0	5.3	4.8	1.7	2.5	1.0
1/3 SW-CMA		0.6	2.4	1.5	1.8	10.2	10.5	0.7	1.6	14.5
1/2 SW-CMA	0.6	1.3	2.7	1.9	1.7	10.1	9.0	1.7	2.9	8.3
1/2 SW-CMAG	0.5	1.1	3.3	2.0	1.3	10.6	9.5	1.8	2.7	7.8

* Growth of less than 0.1 mm/day.

L-1: *Lagenidium callinectes* from *Callinectes sapidus* in North Carolina (Bland and Amerson, 1973).

L-6: *Lagenidium callinectes* from algae in Washington (by Gotelli in 1973).

L-3b: *Lagenidium callinectes* from *Penaeus setiferus* in Texas (Lightner and Fontaine 1973).

L-815: *Lagenidium callinectes* from *Penaeus stylirostris* in Mexico (by Lightner in 1976).

CE: *Lagenidium callinectes* from *Penaeus monodon* in the Philippines (by Gacutan in 1978).

L-F2: *Lagenidium callinectes* from *Pandalus platyceros* in California (by Fisher in 1975).

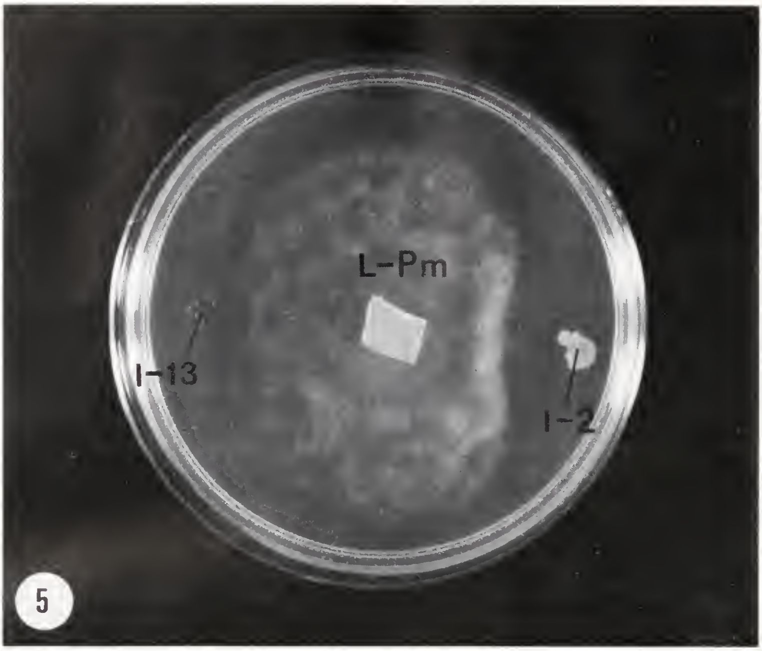
L-Pm: *Lagenidium callinectes* from *Palaemon macrodactylus* in California (described here).

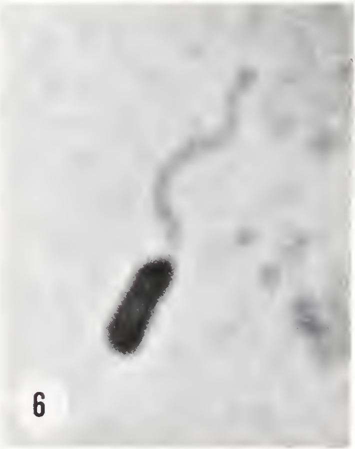
H-2: *Haliphthoros milfordensis* from *Penaeus setiferus* in North Carolina (Tharp and Bland, 1977).

H-222: *Haliphthoros milfordensis* from *Homarus americanus* in California (Fisher *et al.*, 1975).

S-At: *Saprolegnia sp.* from *Acipenser transmontanus* in California (by Fisher in 1981).

Media are described in text.





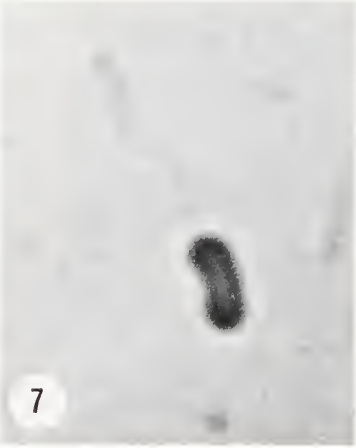
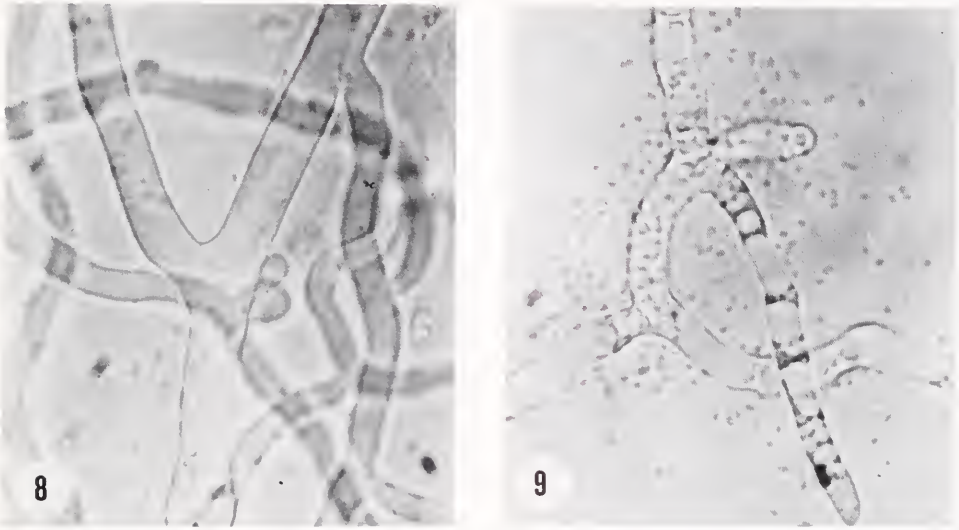


FIGURE 5. Bacterial isolates I-13 and I-2 on agar media (1/3 MA) restricted the growth of the fungus L-Pm growing from a fungal slab transfer in the center of the plate. I-13 and I-2 formed zones of inhibition of 4–8 mm and 6–10 mm respectively.

FIGURES 6 and 7. Flagella stain revealed both I-13 (Fig. 6) and I-2 (Fig. 7) had a single polar flagellum. Straight or slightly curved rods estimated at 2.2 × 0.6 μm with an extended flagellum of 4.4 μm, these marine bacteria are believed to belong to the genus *Alteromonas*. (8,250×).

NaCl concentration, and grow even faster with the balanced salts provided by 1/3 SW-CMA and 1/3 MA. Growth rates decrease as concentrations approach either fresh water or the salt equivalents of full-strength sea water (2.5% NaCl or KCl).



FIGURES 8 and 9. A small agar slab of growing L-Pm was placed in a drop slide (Fig. 8; 1,350×), then inoculated with I-2 bacteria and examined under a compound light microscope. After two days (Fig. 9; 1,340×), there were cavities formed in the hyphal cytoplasm. After six days (not shown), the fungal hyphae were completely devoid of cytoplasm. No stain.

Antagonistic effects of bacteria

There were 177 bacterial isolates obtained from the duplicate 10^{-2} dilution plates from embryo samples of fifteen females taken from their natural environment. Of these, 27 (15%) restricted fungal growth at least 1 mm from the edge of the bacterial colony. An additional 18 (10%) retarded fungal growth by slowing the rate or decreasing the density of the hyphae.

Two bacteria, I-2 and I-13, isolated from embryos on females held in the laboratory system, restricted growth of L-Pm on 1/3 MA plates (Fig. 5). Zones of inhibition were 6–10 mm and 4–8 mm, respectively. Growth of L-Pm from agar slabs in broth (1/3 MB) was luxuriant and easily observable after only 1 day, whereas there was no growth in broth inoculated with I-2 or I-13 bacteria. These bacteria are shown in Figures 6 and 7.

Fungal growth across Nalgene Nutrient Pad Kit membranes, hydrated with sterile 1/3 SW, was thick and luxuriant, reaching a 1–2 mm radius the first day and increasing to over 4 mm by the third day. Fungal slabs retrieved from the membrane, trimmed, and plated on 1/3 MA showed 6–10 mm growth in 24 hours. No growth, however, was recorded after 3 days by fungi on nutrient pads hydrated with broth culture of I-2 bacteria. Fungal slabs retrieved from this treatment after 1 day grew 6–8 mm when plated on 1/3 MA for 24 hours, but those retrieved after 2 days grew only 0–2 mm in 24 hours and those retrieved after 3 days did not grow even after 48 hours on 1/3 MA.

Figures 8 and 9 show the degradation of L-Pm by bacterial isolate I-2. Addition of the bacteria to the growing fungus appeared to cause cavity formation in the hyphal cytoplasm by 2 days (Fig. 9). Six days after the addition of bacteria, fungal hyphae were completely devoid of cytoplasm. This result was verified with stained

TABLE III

Metabolic characteristics of the bacteria I-2 and I-13 compared to recorded characterists of Alteromonas haloplanktis *strain 215*[1] *(A215) and* A. undina *strain 272*[2] *(A272)*

Metabolic characteristics	I-2	I-13	A215	A272
Utilizes:				
Adonitol	−	−	−	−
Citrate	+	+	+	−
Erythritol	−	−	−	−
Fructose*	+	+	+	−
Fumarate	+	+	+	+
Galactose	−	−	−	−
Glucose*	+	+	+	+
Inulin	−	−	−	−
L-Arabinose	−	−	−	+
Lactose	−	−	−	−
m-hydroxybenzoate	−	−	−	−
Maltose	+	−	+	+
Mannose	−	−	+	−
Melibiose	−	−	−	−
N-acetylglucosamine	+	+	+	+
Rhamnose	−	−	−	−
Sorbitol	−	−	−	−
Succinate	+	−	+	+
Sucrose*	+	+	+	+
Trehalose	+	+	−	+
Xylose	−	−	−	−
Produces:				
Alginase	−	−	−	−
Amylase	+	+	−	+
Chitinase	+	+	−	+
Gelatinase	+	+	+	+
Lipase	+	+	+	+

* Negative results for fermentation test.
[1] Baumann and Baumann, 1981.
[2] Chan *et al.*, 1978.

sections. Scanning electron microscopy revealed no visible damage to the exterior surface of the hyphal wall.

Characteristics of the bacteria

Characteristics of the bacterial isolates are shown in Table III. The Gram-negative, marine (requires Na^+), non-fermentative, straight or slightly curved rods of I-2 are motile and form glossy, circular, opaque colonies on 1/3 MA within 3 days at 25°C. I-13 shows similar characteristics except colonies are dark brown and adhere tightly to an agar surface. Both isolates produce extracellular enzymes, including amylase, gelatinase, lipase and chitinase, and each has a single polar flagellum (Figs.

TABLE IV

Distance in millimeters between colonies of I-2 bacteria and the nearest growth of L. callinectes *(strains L-6, L-3b, L-815, CE, and L-F2),* H. milfordensis *(H-2 and H-222),* Saprolegnia *(S-At) and the present isolate L-Pm plated on agar plates of different media*

Media	L-6	L-3b	L-815	CE	L-F2	L-Pm	H-2	H-222	S-At
1/3 MA	2	8	7–10	9	10	7			2
SIA	10	10	8–10	3–8	11	7			2
1/2 SW-CMA	5	4–8	8–9	9	1/0*	1/0*	2/0*	3/0*	2
1/2 SW-CMAG	5	4–9	5–10	9–10	1/0*	1/0*	3/0*	2/0*	2

* Fungal growth was initially inhibited (first number) but then gradually grew over the zone of inhibition to make contact with the bacterial colony (zone of inhibition then equals 0 mm, the second number).

Media are described in the text and fungal isolates are keyed in Table II.

6, 7). These characteristics place them among the marine heterotrophs of the genus *Alteromonas.*

Bacterium I-2 restricts or temporarily inhibits all the fungi tested (Table IV). L-Pm and L-F2 were restricted 7 and 11 mm away from the bacterial colony when plated on 1/3 MA or SIA. However, when they were plated on 1/2 SW-CMA or 1/2 SW-CMAG, only temporary inhibition occurred, slowing the fungal growth within 1 mm of the bacterial colony for a few days, but eventually allowing it to touch. Similar temporary inhibition of L-F2, L-Pm, and S-At was exhibited by *Alteromonas* strains 261, 272, 215, 107 and 113 on 1/3 MA. No inhibition was shown by *Alteromonas* strains 40, 121, and 8 against these same fungi. The presumptive *Alteromonas* isolates, I-2 and I-13, restricted fungal growth several millimeters from their respective colonies (Fig. 5).

DISCUSSION

Externally brooded eggs of *P. macrodactylus* were found susceptible to a fungal pathogen presumptively identified as *Lagenidium callinectes.* Infection always resulted in death, but was observed only on eggs detached from the female or on eggs attached to females with excised first pereiopods. This implied that the first pereiopods of the female played an important role in the defense against fungal infection, either by brushing away fungal propagules or by removing infected eggs to protect healthy eggs in the clutch. Cleaning by the pereiopods has also been shown to reduce bacterial association with the egg surfaces (Fisher, 1983). In a similar study, Bauer (1979) found a higher incidence of particulate debris, bacteria, nematodes, hypotrichous ciliates and dead eggs in clutches of the caridean *Heptacarpus pictus* when the first pereiopods were excised.

Fertilized eggs in this study were less susceptible to fungal infection than unfertilized eggs. This may have been the result of a hardened outer embryonic coat, the fertilization membrane, formed during the fertilization reaction (Cheung, 1966). The harder coat may resist fungal penetration and thereby limit infection. In addition, hyphae seemed unable to penetrate mature (10- to 12-day-old) embryos and, instead, formed a mat around them. Death in this case was probably caused by asphyxiation rather than tissue destruction. Lack of penetration in these older embryos may also be due to increased hardness of the outer embryonic coat. Burkenroad (1947) found increasing resistance to cold, concentrated HCl in the outer coat of developing *Palaemonetes vulgaris* embryos and Cheung (1966) claimed that the egg coat of *Astacus pallipes* became considerably harder in older embryos.

Lagenidium infection of adult *P. macrodactylus* occurred in the holding system and resembled *Haliphthoros milfordensis* infection of *Homarus* postlarvae (Fisher *et al.*, 1975) and *Aphanomyces astaci* infection of European crayfish (Unestam and Weiss, 1970). Several sites of infection were observed, but normally in areas of flexible or "soft" exoskeleton (ventral abdomen, joints). Melanization of infected areas occurred in an apparent attempt to restrict the penetration of the fungus. Molting or removal of infected tissue could halt progress of the disease. Fungal propagules can exist in the system without infecting either adults or eggs, infection presumably requiring a portal of entry and/or sufficient nutrient to allow the growth necessary for penetration.

Growth rate and salt concentration comparisons support the presumptive identification of the fungus as *Lagenidium callinectes* (Table II). L-Pm is unquestionably similar to L-F2, a confirmed member of the species and a previous isolate from northern California. The two isolates have similar salt optima (which also compare favorably with earlier studies of L-F2 by Nilson *et al.*, 1976) and are similarly inhibited by the bacterium I-2 (Table IV).

Bacterial isolates I-2 and I-13 are heterotrophic marine bacteria that produce the enzymes amylase, lipase, gelatinase and chitinase (Table III). *Alteromonas* are the only known heterotrophic marine eubacteria that produce extracellular enzymes (Baumann and Baumann, 1981) and *A. haloplanktis* and *A. undina*, two species from northern California, closely resemble the metabolic characteristics thus far determined for I-2 and I-13 (Table III). Tested strains of *A. haloplanktis* and *A. undina* did not, however, inhibit fungal growth with the same potency of I-2 or I-13.

Bacterial isolate I-2 inhibited growth on agar plates of several strains of *L. callinectes* and *H. milfordensis*, two pathogens of marine Crustacea (Table IV). It also inhibited a freshwater fungus (*Saprolegnia sp.*) isolated from infected sturgeon eggs (*Acipenser transmontanus*). *Saprolegnia* has been recognized as a pathogen for a wide range of fish species (Neish and Hughes, 1980), including the commercially important salmonids, cichlids and anguillids. Successful isolation and characterization of the active substance produced and released by this bacterial isolate may substantially aid in the artificial culture of marine crustaceans and freshwater fishes.

Fungal inhibition has been demonstrated by I-2 on agar plates and on nutrient pads across filter membranes, indicating that the active agent is extracellular. Chitinase was released extracellularly by both I-2 and I-13 and, since it was found to aid in lysing the cell wall of the fungus *Aspergillus* (Horikoshi and Iida, 1959), it may play a role in the antifungal action. Other species of *Alteromonas* which release chitinase did not, however, significantly restrict the growth of L-Pm. Nor did scanning electron microscopy reveal any external deterioration of the fungal wall after incubation with I-2.

The ecological significance of antifungal bacteria inhabiting the surfaces of externally brooded embryos of *P. macrodactylus* has not been investigated. Bacterial type I-2 was isolated during an experiment where penicillin-treated embryos, exhibiting high counts of penicillin-tolerant bacteria, contracted an infection of L-Pm. I-2 was isolated from the untreated, uninfected control embryos and found to be both antifungal and susceptible to penicillin. This implied that I-2 or similar bacteria were protecting the embryos from fungal infection. The high percentage (25%) of bacteria from nature with antagonistic properties supports this possibility and the potent effects of I-2 on a variety of fungi indicates that antifungal bacteria may have a wide range of influence.

The results of this study demonstrate the importance of the cleaning chelipeds in protecting embryos against fungal infection. How decapods without the ability to preen their brooding embryos (the Dungeness crab, *Cancer magister,* for example) protect them from fungal infection is not known. Susceptibility to fungal infection, unlike susceptibility to bacterial infestation (Fisher, 1983), is affected by fertilization and embryonic age. Moreover, the presence of antifungal bacteria on egg surfaces opens the possibility of a wide range of microbial actions and interactions that could affect the survival of brooding embryos.

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GROWTH STUDIES WITH BACTERIA-FREE OYSTER (*CRASSOSTREA GIGAS*) LARVAE FED ON SEMI-DEFINED ARTIFICIAL DIETS[1]

C. J. LANGDON

College of Marine Studies, University of Delaware, Lewes, DE 19958

ABSTRACT

By the aseptic removal of gametes and *in vitro* fertilization of *Crassostrea gigas* eggs it was possible to obtain axenic larvae without the use of antibiotics. Optimal culture conditions for maintaining axenic larvae on algal diets have been developed.

Based on the results of ten growth experiments, *C. gigas* larvae fed on a semi-defined biphasic artificial diet had a mean shell length of 96.0 μm (standard deviation ± 3.0 μm) after 6 days of growth. This was significantly greater ($P < 0.001$) than the mean shell length of starved larvae (79.8 ± 0.8 μm), but was significantly less ($P < 0.001$) than that of larvae fed on the alga *Chaetoceros calcitrans* (113.6 ± 8.0 μm). *C. gigas* larvae grew on the dissolved organic fraction of the artificial diet alone, but growth was less than with the complete biphasic diet. This is the first reported demonstration that bivalve larvae can utilize dissolved nutrients for growth under axenic conditions where the possible nutritional contribution of bacteria is completely eliminated.

INTRODUCTION

One of the first steps in determining the nutritional requirements of an organism is the development of a completely defined artificial diet. However, no artificial diets have been reported that will sustain satisfactory growth of marine bivalves (*e.g.*, Winter, 1974; Masson, 1977). Castell and Trider (1974) reported that growth of *Crassostrea virginica* juveniles fed on semi-defined diets was only one-tenth that of animals kept in the sea.

One of the main difficulties in this area of research is the incompletely known but often deleterious effects of bacterial contamination associated with the culture of bivalves on artificial diets, since bacterial activity is increased by the addition of organic nutrients. Masson (1977), for example, observed that bacterial contamination may not only be directly harmful to bivalve larvae but may also cause food particles to form clumps that are too large for ingestion. On the other hand, bacteria may also serve as an undefined food source in nutrition studies (Zobell and Feltham, 1938; Martin and Mengus, 1977) thus making interpretation of the results virtually impossible.

There have been several successful attempts in obtaining axenic bivalve larvae with the use of antibiotics (Hidu and Tubiash, 1963; Millar and Scott, 1967). These techniques have not, however, been adopted by other workers, possibly because of the poor effectiveness of commonly used antibiotics, such as penicillin and streptomycin, against naturally occurring marine bacteria (Helm and Millican, 1977). In addition, other workers have shown that antibiotics may have adverse effects on

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the growth and survival of molluscs (Seneca and Bergendahl, 1955; Chernin and Schork, 1959).

This paper describes a method for obtaining axenic bivalve larvae without the use of antibiotics, together with the results of growth experiments with axenic *C. gigas* larvae cultured on artificial diets.

MATERIALS AND METHODS

Obtaining axenic C. gigas *larvae*

Preliminary sterility tests using Droop's E6 sterility test medium (Droop, 1969) indicated that it was possible to obtain axenic gametes of *C. gigas* by aseptically removing them from the gonads of ripe adults. It was then possible to obtain viable, axenic *C. gigas* larvae by fertilizing the eggs *in vitro.*

The following protocol was developed in order to obtain axenic larvae for the nutrition experiments: Adult *C. gigas* were conditioned at 21°C for 1 month (Walne and Helm, 1974). After this period, the oysters were opened and a ripe male and female were selected. Under aseptic conditions, the surface of the gonad was wiped with 0.5% hypochlorite solution. A small incision was then made in the wall of the gonad and the gametes removed using a sterile Pasteur pipette. The eggs and sperm were collected separately in flasks containing 100 ml of autoclaved sea water at 25°C and 28 ppt salinity. The eggs, at a concentration of 50 eggs ml^{-1}, were fertilized by adding 100 to 300 sperm ml^{-1}. After fertilization, the eggs were incubated at 25°C for 18 to 24 h. The axenicity of the larvae was tested by adding a few milliliters of the larval suspension to one-tenth concentration of Droop's sterility test medium and incubating the medium at 25°C for 1 month. The development of a bacterial population in the sterility test medium indicated contamination of the larval culture. Eighty-five percent of more than one hundred batches of larvae, were shown to be axenic. The sterility of larvae obtained in this way was confirmed by epifluorescent microscopy using an acridine orange staining technique (Hobbie *et al.,* 1977). The axenicity of the larval cultures was also routinely tested at the end of each feeding experiment with Droop's sterility test medium and only the growth data from axenic cultures were used in the evaluation of the diets.

Axenic culture conditions

Larvae were cultured in the dark, in 100 ml flat bottomed flasks containing autoclaved natural sea water at 25 ppt salinity and 25–28°C. The cultures were agitated to maintain the artificial food particles in suspension. Bubbling was found to adversely effect the growth of larvae in the 100 ml culture flasks compared with the growth of larvae in standing flasks. Helm and Spencer (1972) have also reported a negative effect of bubbling on the growth of early straight-hinged *C. gigas* larvae. Best larval growth occurred in flasks which had a small conical protuberance in the center of the base and were agitated on an orbital shaker at 75 to 100 revs. min^{-1}. The protuberance prevented settled larvae and food from accumulating in the center of the base. Under these conditions, the mean shell length of larvae increased from 76.2 μm to 193.4 μm after 10 days of feeding on an algal diet of *Pyramimonas virginica,* compared with a final mean shell length of 118.0 μm for larvae grown in non-agitated flasks; the difference between the final mean shell lengths was statistically significant ($P < 0.001$).

For the growth experiments with artificial diets, the flasks were first baked at 450°C for 24 h to remove traces of organic material. The flasks were filled with 90 ml of sea water (25 ppt salinity), autoclaved at 115° for 15 min and then agitated on an orbital shaker for 48 h to restore the pH to 8–8.2 and oxygen and carbon dioxide concentrations to normal sea water levels. Larvae were added to the flasks to give a concentration of 2 larvae ml^{-1} and then the artificial diets were added. The cultures were then agitated in the dark on an orbital shaker at 25–28°C. Food particles (2.2–12.6 μm in diameter) were added to the flasks periodically to maintain a particle concentration of between 20 and 100 particles μl^{-1}. The growth experiments were carried out over a period of 6 days. The culture medium was not changed during the experimental period.

At the end of the experiment, the larvae were sieved from the culture medium using a 35 μm mesh screen and preserved in 1% formalin solution (made up in 25 ppt salinity sea water, pH 8.0). The shell lengths of at least 100 larvae from each culture were measured. The mean shell length and standard deviation were calculated and comparisons among treatment means were made using Student's *t*-test.

Preparation and development of an artificial diet

A biphasic diet was developed for *C. gigas* larvae which consisted of a soluble phase and a particulate phase, the latter made up of co-precipitated egg albumin and starch particles. Biphasic diets have been used successfully in the axenic culture of the brine shrimp *Artemia salina* (Provasoli and d'Agostino, 1969) and the water flea *Moina macrocopa* (Conklin and Provasoli, 1977, 1978). The gross composition of the particle developed for *C. gigas* larvae was based on the composition of the alga *Pavlova lutheri*, as reported by Parsons *et al.* (1961), which is a satisfactory algal food species for *C. gigas* (Millican and Helm, 1973). The composition of the particle is given in Table 1.

The particle was prepared by firstly heating and dissolving 150 mg of rice starch in 20 ml of distilled water. The solution was placed on a magnetic stirrer, a stream of nitrogen was introduced, and 50 mg of an oyster lipid extract, dissolved in 2 ml of chloroform, were added. The lipid extract was prepared by homogenizing the body tissues of adult *C. gigas* in chloroform/methanol according to the method of Bligh and Dyer (1959). The chloroform was removed with gentle heating. Next 59.3 mg of $Na_2HPO_4 \cdot 2H_2O$ (equivalent to 10 mg of phosphorus), 200 mg of egg albumin (Sigma, Fraction V) dissolved in 5 ml of 1 *M* NaCl, together with 1 mg each of RNA (Sigma, from yeast) and DNA (Sigma, from salmon sperm) dissolved in a minimum volume of 1 *M* NaOH were added. The mixture was poured into 70 ml of boiling sea water in order to precipitate the particle, and the pH of the suspension adjusted to pH 8.0 with 1 *M* NaOH. The precipitate was then autoclaved and a fine particulate suspension was prepared by homogenization with a sterile glass tissue grinder under aseptic conditions.

The composition of the water-soluble phase of the diet was initially based on that of tissue culture medium (TCM) 199 and was prepared according to Morgan *et al.* (1955). All the soluble nutrients of TCM 199 were sterilized by 0.2 μm Millipore filtration. It was found that the concentrations of the dissolved nutrients of TCM 199, recommended for the culture of animal tissues, were too high for oyster larvae and caused both swelling and protrusion of the velum from between the shell valves. This condition resulted in tissue loss and death of the larvae. At one-hundredth the recommended concentration of TCM 199, larvae grew without the development of such morphological abnormalities.

In subsequent experiments, several alternatives to the TCM 199 amino acid mixture were tested at different concentrations. These mixtures were based on the

TABLE I

Composition of the final artificial diet developed for axenic Crassostrea gigas *larvae*

Co-precipitated particle. Fed at a concentration of 100 particles μl^{-1}

	Composition, ratio by wt.
Egg albumin (Fraction V, Sigma)	200
Rice starch	150
Oyster lipid extract	50
Phosphorus (as $PO_4^{///}$)	10
R.N.A. (yeast)	1
D.N.A. (salmon sperm)	1

Dissolved nutrients

[a] *Amino acids*	*mg* l^{-1}
Alanine	0.97
DL-2-amino-iso-butyric acid	0.05
DL-2-amino-n-butyric acid	0.13
Arginine (Cl)	0.57
Aspartic acid	0.99
Cysteine (Cl)	0.05
Glutamic acid (H_2O)	0.84
Glycine	0.63
Histidine (Cl)	0.19
iso-Leucine	0.33
Leucine	1.02
Lysine (Cl)	0.73
Methionine	0.32
DL-Ornithine	0.04
Phenylalanine	0.44
Proline	0.67
Serine	0.60
Threonine	0.50
Tryptophan	0.04
Tyrosine (Na_2 salt)	0.21
Valine	0.68

Glucose, water and fat-soluble vitamins, purines, pyrimidines and other water soluble components were added at 1/100 the concentration recommended for tissue culture medium 199 (Morgan *et al.*, 1955).

1% v/v bovine amniotic fluid (Gibco) was added as a beneficial, undefined source of nutrients.

[a] Amino acid mixture based on the composition of the alga *Isochrysis galbana*, according to Chau *et al.* (1967). All amino acids were L-isomers unless otherwise indicated.

composition of the culture medium for *A. salina* (AA 1A mix; Provasoli and d'Agostino, 1969) and for *M. macrocopa* (Conklin and Provasoli, 1977, 1978) as well as a mixture based on the composition of the alga *Isochrysis galbana* (after Chau *et al.*, 1967). Of these, the *Isochrysis* mixture (see Table I), at a total amino acid concentration of 10 mg l^{-1}, resulted in significantly greater larval growth ($P < 0.001$) than with the other mixtures tested. It was therefore used instead of the TCM 199 amino acid mixture in the artificial diet. The amino acid mixture developed for *A. salina* was harmful to the oyster larvae at the concentration used by Provasoli and d'Agostino (1969), and caused swelling of the velum and tissue loss.

A range of water soluble vitamin mixtures was also tested to find substitutes for the TCM 199 vitamin mixture at different concentrations. These included Conklin and Provasoli's (1977, 1978) mixture for *M. macrocopa* and the ASP_2 vitamin mixture reported by Provasoli *et al.* (1957) for the culture of marine algae. Neither of these was superior to the TCM 199 vitamin mixture at one-tenth the recommended concentration, and the vitamin mixture described for *M. macrocopa* was harmful to oyster larvae both at one-tenth and at the full concentration reported to support good growth of this crustacean species. Later experiments indicated that the best larval growth occurred if the concentration of the water soluble vitamins of the TCM 199 medium was reduced from one-tenth to one-hundredth that of the recommended concentration for the culture of mammalian cells (Morgan *et al.*, 1955).

Apart from the defined nutrients of the culture medium developed for oyster larvae, it was found that the addition of 1% v/v bovine amniotic fluid (Gibco) to the artificial diet significantly improved larval growth ($P < 0.05$). This undefined nutrient source was commercially available as a sterile solution. The composition of the final oyster culture medium is given in Table 1.

RESULTS

Growth of larvae fed on the final artificial diet of Table I

Based on the results of ten separate experiments, larvae fed on the final artificial diet (Table I) had a mean shell length of 96.0 μm (standard deviation ± 3.0 μm) after 6 days of growth. This was significantly greater ($P < 0.001$) than the mean shell length of starved larvae (79.8 ± 0.8 μm), but was significantly less ($P < 0.001$) than that of larvae fed on the alga *Chaetoceros calcitrans* (113.6 ± 8.0 μm). Most of the larvea fed on the artificial diet reached the umbone stage of development within 6 days, but little further growth occurred, and after 8 days tissue loss became increasingly apparent. Feeding experiments indicated that the larvae fed on the artificial diet cleared very little of the suspended particulate fraction, and the guts of approximately 80% of the larvae were empty.

Larvae fed on mixtures of algae and artificial nutrients

It was difficult to determine whether the poor feeding activity of the larvae on the artificial food particles was due to deficiencies in the nutritional quality of the diet or due to some growth inhibitory effect of the dietary components. An experiment was therefore carried out in which the particulate and dissolved fractions of the diet were tested singly or in combination with an algal diet. Any inhibitory effect of either of these two fractions on the growth of the larvae would, therefore, become evident. For this experiment, *Dunaliella tertiolecta* was chosen as the algal food since it was available in axenic culture and was reported to be of only moderate food value for oyster larvae (Walne, 1963); therefore, there was possible scope for improvement of the algal food by the addition of supplements of artificial nutrients.

The results (Table II) indicated that in the absence of *D. tertiolecta* the larvae grew best on the complete artificial diet. Larvae grown on the dissolved organic fraction alone were significantly larger ($P < 0.001$) than starved larvae after 6 days of culture. The particulate fraction of the diet did not support larval growth and tissue loss and high larval mortality was evident. In combination with *Dunaliella*, the complete artificial diet had little effect on larval growth compared with the growth of larvae fed on *Dunaliella* alone. However, addition of the dissolved organic fraction of the artificial diet to *Dunaliella*, significantly improved larval growth ($P < 0.001$) compared with the growth of larvae fed on algae alone. Addition of the

TABLE II

Growth of axenic C. gigas *larvae fed on components of the artificial diet (Table I) alone or in combination with the alga* Dunaliella tertiolecta *(25 cells μl^{-1})*

Artificial diet	[a] Mean shell length ± s.d. (μm)	
	Without algae	With algae
None	80.9 ± 0.8	97.1 ± 1.2
Particulate fraction	Larvae died, vela protruding	Larvae died, vela protruding
[b] Dissolved fraction	95.3 ± 1.0	123.6 ± 2.3
Dissolved plus particulate fraction	98.9 ± 0.8	97.7 ± 1.2
Initial larval shell length = 74.7 ± 0.5 μm.		

[a] Based on two replicate cultures for each treatment and 100 larvae measured from each culture.

[b] Dissolved organic fraction consisted of the defined components given in Table I plus 1% v/v bovine amniotic fluid.

particulate fraction of the diet to *Dunaliella* caused protrusion of the vela and high larval mortality.

Testing alternative food particle types to the co-precipitated egg albumin and starch particles

The results of the growth and feeding experiments indicated that the co-precipitated egg albumin and starch particle was not a satisfactory means of feeding the larvae on protein and carbohydrate. Therefore a range of alternative food particle types were tested (see Table III). Red blood cells were selected for testing because Claus and Adler (1970) reported good growth of both *C. virginica* larvae and spat fed on this food. Since Conklin and Provasoli (1977, 1978) reported the successful

TABLE III

Growth of C. gigas *larvae cultured on the dissolved organic fraction of the artificial diet (Table I) and particulate foods*[a] *under axenic conditions*

Diet	Mean shell length + s.d. (μm)
Controls	
Starved larvae	79.9 ± 0.8
Algal-fed larvae[b] (non-axenic)	99.6 ± 1.8
Dissolved organic fraction (DOF) alone	87.7 ± 1.1
DOF + egg albumen/starch particles	94.3 ± 1.2
Treatments	
DOF + defibrinated blood cells (calf)	86.7 ± 1.1
DOF + bovine serum albumin/amylose particles	90.6 ± 0.9
DOF + heat precipitated bovine amniotic fluid[c]	90.7 ± 0.9
DOF + raw whole egg particles	91.9 ± 1.1[d]
DOF + raw egg yolk particles	94.0 ± 1.1[d]
Initial larval shell length	75.4 ± 0.4 μm

[a] All particles were added at concentrations of 100 particles μl^{-1}.

[b] Algal food was *Chaetoceros calcitrans* at an initial concentration of 100 cells μl^{-1}.

[c] Added at a concentration of 1% v/v.

[d] >75% larvae were in poor condition with protruding vela and loss of body tissues.

use of particles prepared with egg yolk or bovine serum albumin in the culture of *M. macrocopa*, particles prepared with these nutrients were also tested. The bovine serum albumin/amylose particles were prepared by co-precipitation with heat in a similar way to the egg albumin and starch particles (see Methods section).

None of the food particle types supported larval growth better than the co-precipitated egg albumin and starch particles ($P < 0.05$) (Table III). Furthermore, both whole egg and egg yolk had an adverse effect on the larvae and caused loss of the body tissues and protrusion of the velum. Larval growth on the dissolved organic fraction of the diet alone was statistically greater ($P < 0.001$) than that of starved controls, and was further improved by adding the egg albumin and starch particles to the dissolved organic fraction; these results therefore confirmed the findings of the previous experiment (Table II).

DISCUSSION

A method for reliably obtaining large numbers of axenic *C. gigas* larvae without the use of antibiotics has been described. The method need not be restricted to oysters since it can be applied to animals from which axenic gametes can be removed and fertilized *in vitro* under aseptic conditions. The ability to eliminate bacteria from nutrition studies of marine filter-feeders is an essential step in the development of defined artificial diets for these animals. This method may also be useful in studies of the interaction between marine invertebrates and bacteria.

The feeding experiments indicated that the culture media developed for *A. salina* (Provasoli and d'Agostino, 1969) and *M. macrocopa* (Conklin and Provasoli, 1977, 1978) was not suitable for *C. gigas* larvae since the larvae would not tolerate high concentrations of the dissolved nutrients. Even so, the lower concentrations of the dissolved nutrients of the artificial diet developed for *C. gigas* were much higher than those occurring in natural conditions. For example, the concentration of free amino acids in the dissolved organic fraction of the diet (10 mg l^{-1}) was about one thousand times greater than those reported to occur naturally in oceanic waters (Williams, 1975). Furthermore, co-precipitated egg albumin and starch particles, which were similar to those developed for *A. salina* and *M. macrocopa*, caused protrusion of the velum, tissue loss, and high mortality when fed to the larvae in the absence of the dissolved organic fraction of the diet (Table II). However, in combination with the dissolved organic fraction the particles had a positive effect on growth (Table II and III), although very little ingestion of the particles was apparent.

It is not clear why the adverse effect on the larvae of the co-precipitated egg albumin and starch particles depended on the absence of the dissolved organic fraction of the diet (Table II). Adverse effects were also evident with raw whole egg and raw egg yolk (Table III) as well as with other nutrients tested but not reported here such as calf serum, lobster haemolymph, casein, and freeze dried or heat killed algal cells (see Langdon, 1980). All these nutrients were similar in that they were largely or wholly made up of protein. It is probable that particulate or dissolved foreign proteins had a toxic effect on larvae which was apparent in the protrusion of the velum and high mortality. Further testing of this hypothesis is necessary.

The dissolved organic fraction of the artificial diet (Table I) supported larval growth that was significantly greater ($P < 0.001$) than that of starved controls (Table II, III). This is the first demonstration that marine bivalve larvae can utilize dissolved nutrients for growth under axenic conditions where the possible nutritional contribution of bacteria is completely eliminated.

There is now conclusive evidence that there is a net uptake of dissolved amino acids from sea water at naturally occurring concentrations by adult mussels *Mytilus edulis* (Manahan *et al.*, 1982). Manahan and Crisp (1982) have also demonstrated that *C. gigas* larvae take up dissolved ^{14}C-labelled amino acids from sea water and either respire the ^{14}C as $^{14}CO_2$ or incorporate the ^{14}C into macromolecules in the body tissues. Uptake is via the velum and not via the gut (Manahan and Crisp, 1982) and therefore, in oyster larvae, uptake of nutrients via the velum compliments uptake of the nutrients via the gut.

The beneficial growth effect of the dissolved organic fraction of the artificial diet was also evident when it was added as a supplement to the alga *Dunaliella*, compared with the growth of larvae fed on *Dunaliella* alone (Table II). Whether larval growth was enhanced directly by uptake of the dissolved organic fraction or indirectly via prior algal uptake, is not known. Davis and Chanley (1955) also reported improvement in the growth of *C. virginica* and *Ostrea edulis* larvae fed on an algal diet supplemented with a vitamin mixture. D'Agostino and Provasoli (1968) found an improvement in the growth and development of *A. salina* when vitamin supplements were added to algal diets and Murphy (1970) noted similar findings with various species of *Daphnia*.

In conclusion, *C. gigas* larvae required both particulate and dissolved organic nutrients for maximum growth when fed on the artificial diet tested. Further research is required, however, in order to improve the methods of delivering dietary components to the larvae. Co-precipitated egg and starch particles are not satisfactory since they are poorly ingested and have an inhibitory effect on growth when fed to the larvae in the absence of the dissolved organic fraction of the diet. Recent techniques for delivering dietary protein in alginate or carboxymethyl cellulose gel particles (Langdon, 1983) are proving to be advantageous. When a satisfactory method for delivering nutrients to bivalve larvae is found then the effects of the nutritional composition of artificial diets on oyster growth can be tested with greater rigor. With axenic culture conditions, it will be possible to chemically specify the nutritional requirements of bivalve larvae and to better understand their nutrition in both natural and artificial conditions.

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THE UPTAKE AND METABOLISM OF DISSOLVED AMINO ACIDS BY BIVALVE LARVAE

DONAL T. MANAHAN

N.E.R.C. Unit of Marine Invertebrate Biology, Marine Science Laboratories, Menai Bridge, Anglesey, North Wales LL59 5EH, U. K.

ABSTRACT

The rates of uptake and metabolism of ^{14}C-labeled glycine and alanine from sea water into larval oysters, *Crassostrea gigas* (Thunberg) and mussels, *Mytilus edulis* L. were determined. Kinetic studies showed that both species have a K_t value of 3–4 μM, indicating that bivalve larvae have amino acid transport mechanisms that function efficiently in natural sea water. The K_t values for larvae are similar to those reported for adult bivalves. However, larvae take up dissolved amino acids at approximately ten times the rate reported for adult bivalves on a gram dry weight basis. This difference in uptake capacity presumably reflects the greater absorptive surface area to volume ratio of a larva. Rates of metabolism of absorbed amino acids by larvae were also rapid. Following a 100 min exposure, oyster larvae incorporated 47% of the glycine into protein and 38% was produced as CO_2. In comparison to adults, larval bivalves have a more rapid weight-specific uptake and faster rate of utilizing absorbed amino acids. Dissolved nutrients may be of vital importance to larvae when particulate food is scarce since they are often provided with minimal food reserves by the parent.

INTRODUCTION

The possibility that dissolved organic material (DOM) in sea water may contribute to the nutrition of marine invertebrates is an attractive idea, since dissolved organic carbon (circa 3 mg C/l; Williams, 1975) is present at concentrations approximately ten times that of particulate organic carbon (circa 0.2 mg C/l; Parsons, 1975). During this century there have been many studies on the uptake of DOM directly from sea water by marine invertebrates (see reviews by Krogh, 1931; Jørgensen, 1976; Stewart, 1979; Stephens, 1981). Studies to date have demonstrated that ^{14}C-labeled substrates, such as amino acids and sugars, are removed from dilute solution in sea water by soft-bodied marine invertebrates and that uptake occurs across the body wall or via specialized organs such as the ctenidia of molluscs. The relationship between uptake and substrate concentration is saturable, indicating a carrier-mediated process typical of active transport systems.

Nearly all of the studies examining the role of DOM in animal nutrition have been carried out using adult invertebrates. However, there are some reports that DOM can be utilized by larval forms. For example, embryos of the ophiuroid *Amphipholis squamata* have a greater capacity for assimilating dissolved glycine and glucose into their tissues than do adult ophiuroids (Fontaine and Chia, 1968). Larvae of the polychaete worm *Nereis virens* absorb 200 times more amino acid per gram

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Present address: Department of Developmental and Cell Biology, University of California, Irvine, California 92717, U.S.A.

Abbreviations: DOM—dissolved organic material.

wet weight than adults (Bass *et al.*, 1969). Reish and Stephens (1969) carried out a detailed investigation on the relationship between body size and the rate of glycine uptake by the polychaete, *Neanthes arenacoedentata.* The linear relation between log uptake and log wet body weight suggested that uptake is dependent upon surface area. Significantly, these authors also found that non-feeding larval stages absorbed glycine at a rate 3–5 times that of feeding stages. Larvae of the bivalve *Mactra* sp. take up ^{14}C-glucose (Crane *et al.*, 1957) and larvae of the Pacific oyster *Crassostrea gigas* have been shown to take up an undefined ^{14}C-labeled algal exudate from sea water (Fankboner and deBurgh, 1978). Rice *et al.* (1980) demonstrated that larvae of the European oyster *Ostrea edulis* absorb dissolved amino acids.

Although there is evidence that bivalve larvae can take up DOM, the contribution of this pathway to their nutritional requirements remains obscure. Manahan and Crisp (1982) demonstrated that the velum of a bivalve larva is capable of absorbing dissolved amino acids directly from sea water. Since the velum presents a large surface area for absorption of dissolved nutrients, bivalve larvae are anatomically well adapted to benefit from this type of assimilation. In this report, I describe kinetic and time course studies on the rates of uptake and metabolism of ^{14}C-labeled amino acids by veligers and pediveligers of the Pacific oyster, *Crassostrea gigas* (Thunberg), and by veligers of the mussel, *Mytilus edulis* L.

MATERIALS AND METHODS

Animals

The routine rearing of larvae was carried out as described by Loosanoff and Davis (1963). Two batches of oyster larvae were used. The *C. gigas* pediveligers were supplied by the Fisheries Experimental Station, Conway, North Wales, where they had been reared at 25°C and a salinity of 25‰. A second batch of *C. gigas* larvae was spawned and reared at Menai Bridge for 20 days (25°C; 32‰) until the larvae had reached the late veliger stage of 300 μm shell length. *M. edulis* larvae were grown at 15°C and 32‰ for 50 days; by then all the larvae had reached the eyed stage.

Incubation media

All glassware was sterilized either by autoclaving at 120°C for 20 min, or by heat-sterilization at 150°C overnight. Plastic sieves used to handle the larvae were pasteurized at 60°C overnight. For experiments, larvae were placed in sterile-filtered (0.2 μm Millipore) sea water at the appropriate temperature and salinity. The labeled amino acids L-(U-^{14}C) alanine (172 mCi/mmol) or (U-^{14}C) glycine (118 mCi/mmol) were added to give the required test concentrations.

Kinetic and time course experiments

Larvae used in uptake experiments were siphoned onto a 45 μm mesh screen to select for uniform size. The larvae were blotted to remove adherent fluid, washed three times with 100 ml of sterile-filtered sea water, and distributed into glass vials at a density of about 40/ml. Two experiments were carried out in parallel for each group of larvae, *C. gigas* pediveligers and *M. edulis* veligers. (a) For kinetic experiments, larvae were exposed for the shortest practicable period (5 min) to a ^{14}C-labeled amino acid ranging in concentration from 0.1 μM to 10 μM; (b) in time course experiments, the larvae were exposed to circa 0.5 μM ^{14}C-labeled substrate for 100 min. Separate vials of larvae were harvested at 0, 10, 20, 40, 60, 80, and

100 min to measure the ^{14}C-label in the larvae. In parallel time course experiments, the production of $^{14}CO_2$ by oyster larvae was measured.

Measurement of $^{14}CO_2$ production

Actively swimming bivalve larvae spend periods of time with their vela close to the sea water/air interface. During this behavior, known as "rafting," any volatile constituent containing ^{14}C might pass directly from the larva into the air. Respired $^{14}CO_2$ could thus be underestimated in samples of medium taken at the end of the experiment. Therefore, a system was developed to trap all the $^{14}CO_2$ produced. Larvae were placed in a 2 ml glass vial which was glued to the base of a 15 ml glass vial. One ml of 10% KOH was pipetted onto the base of the 15 ml vial. The system was sealed by placing a teflon Suba Seal into the opening of the 15 ml vial. One such $^{14}CO_2$ trapping system was set up for each of the 21 determinations made during a 100 min time course experiment. At a given time interval, 0.5 ml of HCl was injected through the Suba Seal into the 2 ml vial containing the larvae, thereby stopping the experiment and increasing the acidity of the sea water to pH 1–2. Any $^{14}CO_2$ present was driven off and trapped in the 10% KOH during the following 16–24 h; thereafter, the vial was opened and the larvae in each one counted. Data could then be expressed as cpm of $^{14}CO_2$ produced per larva.

The efficiency of $^{14}CO_2$ trapping was determined using radioactive bicarbonate standards. A series of $^{14}CO_2$ trapping vials containing 1 ml of standard were set up and acidified. 0.5 ml samples of the initial 1 ml 10% KOH were taken from each vial at various time intervals. The KOH sample was placed in a scintillation vial containing 1 ml methanol and 10 ml Aquasol. The addition of methanol was important, as it prevented the formation of a precipitate when KOH was added directly to Aquasol. By 16–24 h, 95% of the $^{14}CO_2$ was trapped.

Determination of radioactivity in larvae

At each sampling in the time course experiment, larvae were poured from the vial onto a 45 μm mesh sieve and rinsed with 100 ml of sea water. In this condition, the larvae can be stored at 4°C on damp filter paper. Preliminary experiments showed that no decrease in radioactivity per larva occurred during a 5 h storage; in fact, not more than 2 h of storage was ever necessary. The sampling error (95% confidence limits/mean) for the 15 data points of these tests was 5–6% for both mussel and oyster larvae. Three samples of larvae from each sieve were placed on gridded Millipore filters (type: RAWG 02500) and each filter in turn placed on a glass slide and the larvae counted under a microscope. The larvae and filter were placed in a glass scintillation vial (teflon capped) and digested with 1 ml of Tissue Solubilizer (NCS, New England Nuclear) at 50°C overnight. Samples were then bleached to improve radioactive counting efficiency by incubation with 1 ml of 20% benzoyl peroxide in toluene at 50°C for 3 h. After cooling, 10 ml of Aquasol was added to each vial. All samples were stored in the dark for at least 16 h prior to counting. Quench correction was carried out by the addition of internal standards using ^{14}C-hexadecane.

The uniform procedure used to prepare samples for radioactive counting, reduced any differences in quenching caused by digesting different numbers of larvae per sample. Knowing the total radioactivity per sample, the number of larvae in that sample, and the specific activity of the isotope, uptake rates could be expressed as grams ^{14}C-labeled amino acid per larva.

TABLE I

Scheme for determining the percentage of ^{14}C in the lipid, protein, and small molecular weight (TCA soluble) fractions of bivalve larvae

<table>
<tr><th colspan="3">Add 800 μl distilled H_2O to freeze dried sample</th></tr>
<tr><td colspan="3">Homogenate</td></tr>
<tr><td>*Lipid*</td><td>*Protein*</td><td>*Total*</td></tr>
<tr><td>3 × 100 μl
replicate samples:</td><td>3 × 100 μl
replicate samples:</td><td>Single 100 μl
sample:</td></tr>
<tr><td>100 μl sample
+ 100 μl H_2O
+ 250 μl $CHCl_3$
+ 500 μl CH_3OH.
Shake 1 min.
Stand at 4°C 10 min.
Centrifuge 8,000 *g* for 1 min.
Removal 800 μl supernatant (cf. 950 added).
800 μl supernatant
+ 200 μl $CHCl_3$
+ 200 μl H_2O
Shake 1 min.
Centrifuge 8,000 *g* 1 min.
Remove top phase.
Wash bottom phase ×3 with
500 μl Folch rgt.
Dry lower phase at 54°C
Residue = LIPID
+ 500 μl NCS Tissue Solubilizer.
Digest at room temperature overnight.</td><td>100 μl sample
+ 100 μl H_2O
+ 100 μl 15% cold TCA.
Shake 1 min.
Stand at 4°C for 10 min.
Centrifuge 8,000 *g* 1 min.
Remove all of supernatant.
Wash ppt. ×3 with 200 μl 5% TCA removing supernatant each time.
ppt. = Protein
+ 500 μl NCS Tissue Solubilizer.
Digest at room temperature overnight.</td><td>100 μl
+ 500 μl NCS
Tissue Solubilizer.
Digest overnight.</td></tr>
</table>

Biochemical fate of amino acid carbon

In the experiments determining radioactivity in larvae, there always remained a large surplus of larvae available for biochemical fractionation. These larvae were harvested at 10 and 100 min during the time course experiment described above, and were washed onto a GFC filter paper and freeze dried. Using methods modified from Holland and Gabbott (1971), homogenates of these larvae were quantitatively separated into lipids, proteins, and, by subtraction from the total, small molecular weight compounds and carbohydrates (TCA soluble, see Table I). After separation of the fractions, the amount of radioactivity in digests of the various fractions was determined. The total uptake of ^{14}C-labeled amino acid was measured by adding the observed ^{14}C incorporated in the tissue to the $^{14}CO_2$ expired. The former could be proportioned into that metabolized into protein, lipid, and TCA soluble compounds.

Oxygen consumption

The rate of O_2 consumption by *C. gigas* larvae (300 μm shell length) at 21°C and 25‰ was measured with a Radiometer oxygen electrode. The method is described in detail by Davenport (1976).

Results

Crassostrea gigas pediveligers

(a) Kinetics of glycine uptake

Figure 1 shows the effect of increasing substrate concentration on the rate of glycine uptake by *C. gigas* pediveligers. Glycine uptake is evidently concave to the

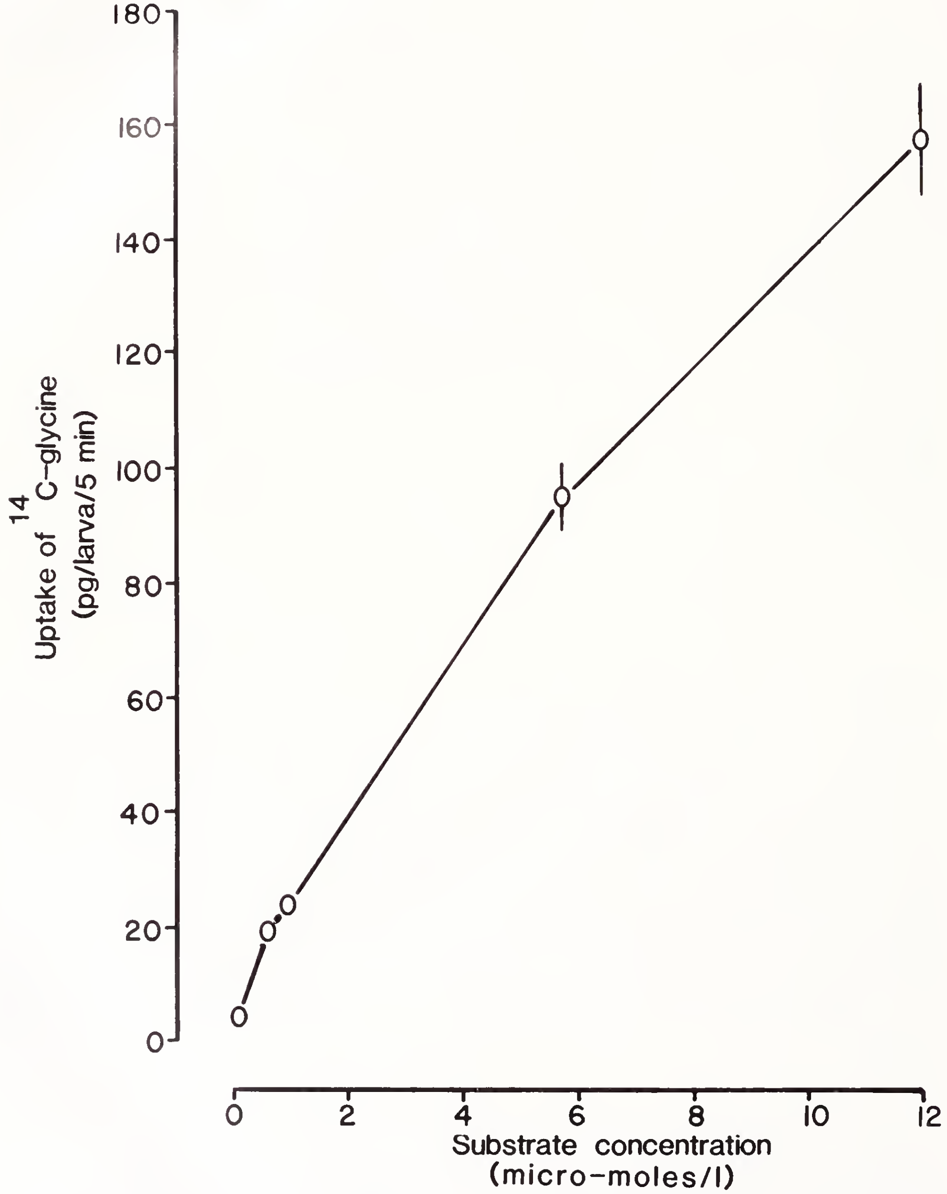

FIGURE 1. The uptake of ^{14}C-glycine by *C. gigas* pediveligers at substrate concentrations ranging from 0.1 μM to 10μM. Temperature = 25°C. Salinity = 25‰. Data points are mean ± S.E. for n = 3. Where no bar errors are shown on a data point, the bars lie within the graphical presentation of the point.

TABLE II

Kinetic constants for amino acid transport by oyster (C. gigas) *and mussel* (M. edulis) *larvae*

Species	Temp.	Salinity	Substrate	$K_t \pm$ S.E.	V_{max}
C. gigas	25°C	25‰	glycine	3.7 $\mu M \pm 1.0$	1.8 ng larva^{-1} hr^{-1}
M. edulis	15°C	32‰	L-alanine	3.5 $\mu M \pm 0.5$	0.9 ng larva^{-1} hr^{-1}

abscissa, and therefore appears to be saturable. A Michaelis-Menten analysis of uptake was made using Hofstee's (1959) linear transformation. The transport constant, K_t, is the substrate concentration at which uptake is experimentally determined to be half the maximum transport capacity of the system (½ V_{max}). Table II gives the kinetic data for *C. gigas* larvae. A low K_t of 3.7 μM was obtained for glycine transport.

(b) *Time course of glycine metabolism*

The time course of ^{14}C-glycine uptake from a concentration of 0.59 μM is shown in Figure 2. Since the numbers of larvae used were in the order of 40/ml, a calculation based on the uptake rates given below shows that total larval uptake would not reduce the substrate concentration by more than 0.02%. The glycine concentration therefore remained virtually constant during the experiment and the linearity of the plots in Figure 2 is to be expected. Regression of uptake in the larva on time gave a rate of 1.68 pg ^{14}C-glycine larva^{-1} min^{-1}; glycine appeared as $^{14}CO_2$ at a rate of 1.02 pg ^{14}C-glycine larva^{-1} min^{-1}. The striking feature of these data is the rapid rate of $^{14}CO_2$ production. A more complete picture of the fate of glycine after absorption by pediveligers is given in Table III. After 100 min exposure, 47% of the glycine carbon had been synthesized into protein and 38% appeared as $^{14}CO_2$. In this short time, less than 2% of the ^{14}C-carbon was found in lipid. As the exposure time to glycine was increased for 10 to 100 min, the amount of glycine metabolized by the larva to lipid, protein, and CO_2 continued to increase by about a factor of 10.

Crassostrea gigas veligers

Figure 3 shows the uptake of glycine by veligers from a concentration of 0.69 μM. $^{14}CO_2$ is also produced from glycine by veligers at a rapid rate of 0.74 pg larva^{-1} min^{-1}, when compared to an uptake rate into the larva of 1.22 pg ^{14}C-glycine larva^{-1} min^{-1}. Table IV gives the more detailed account of glycine metabolism by veligers. By 100 min, 16% of the ^{14}C-label was found in protein and 33% had been recovered as CO_2. Lipid contained less than 1%.

Mytilus edulis veligers

(a) *Kinetics of alanine uptake*

Figure 4 shows the kinetic response of *M. edulis* larvae to increasing alanine concentrations. The kinetic constants obtained from a Hofstee plot of these data are given in Table II. A low K_t for alanine of 3.5 μM was obtained, which is very similar to that of 3.7 μM obtained for glycine uptake by oyster larvae.

(b) *Time course of alanine metabolism*

The uptake of alanine from a concentration of 0.41 μM is shown in Figure 5. There are no data for $^{14}CO_2$ production during this experiment. Table V gives the

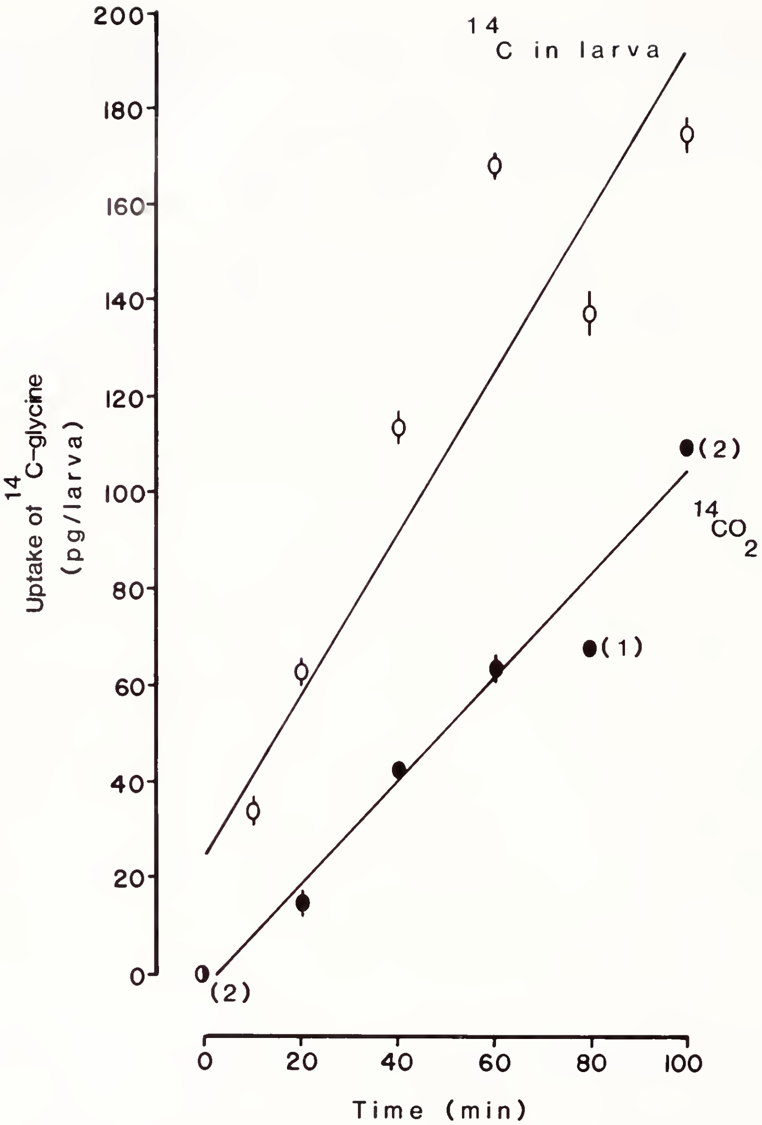

FIGURE 2. Time course of ^{14}C-glycine uptake and oxidation by *C. gigas* pediveligers. ^{14}C-glycine concentration = 0.59 μM. Temperature = 25°C. Salinity = 25‰. Open circles represent ^{14}C recovered in larvae. Closed circles represent $^{14}CO_2$ produced by larvae. All data points are mean ± S.E. for n = 3. Where n is less than 3, the number of replicates is given above the data point. Where no bar errors are shown on a data point, the bars lie within the graphical presentation of the point.

Regression equations

Treatment	*n*	*a*	*b* ± *S.E.*	r^2
Uptake by larva	21	24.10	1.68 ± 0.15	0.86
$^{14}CO_2$ production	17	−2.52	1.02 ± 0.07	0.98

TABLE III

The metabolism of ^{14}C-glycine by C. gigas *pediveligers*

Time		Metabolic fraction (pg ^{14}C-gly/larva)	Metabolic fraction (% of total)
10 min	Total uptake	42.3	
	Lipid	0.9	2.1
	Protein	16.5	39.0
	CO_2	8.4	19.9
	TCA soluble	16.5	39.0
100 min	Total uptake	283.0	
	Lipid	4.2	1.5
	Protein	133.6	47.2
	CO_2	109.0	38.5
	TCA soluble	36.2	12.8

biochemical fate of ^{14}C-alanine following assimilation. A similar pattern to that obtained for oyster larvae is evident. A greater percentage of the ^{14}C-label was present in protein than in lipid, and the amount of assimilated ^{14}C-alanine increases with exposure time.

Oxygen consumption of Crassostrea gigas veligers

The O_2 consumption of *C. gigas* larvae (300 μm) was determined at an early stage of the investigation to be 6.6 nl and 9.5 nl O_2 larva^{-1} h^{-1} for two replicate experiments carried out at 21°C. Assuming a Q_{10} of 2, this oxygen consumption would increase to 10.6 nl O_2 larva^{-1} h^{-1} at 25°C, the temperature of the glycine uptake experiments. This figure accords well with an estimate of 9–11 nl O_2 larva^{-1} h^{-1} for larvae of the European oyster *Ostrea edulis* at the same size (297 μm), based on the value quoted by Crisp (1976) of 5–6 ml O_2 (g dry wt)$^{-1}$ h^{-1}, and on Holland and Spencer's (1973) dry organic weight for a larva of this size (1.84 μg).

DISCUSSION

Rates of uptake of amino acids

The kinetic data for oyster and mussel larvae (Table II) include a K_t of 3–4 μM for each species. This low K_t indicates that bivalve larvae have amino acid transport mechanisms that function efficiently in natural sea water where the free amino acid concentrations lie in this order of magnitude (*e.g.*, North, 1975). The K_t values for the larvae are similar to those reported by Wright and Stephens (1978) for intact adult mussels, *Mytilus edulis* (K_t = 2–5 μM). Hence, both larval and adult bivalves are adapted to utilize dissolved amino acids in sea water.

However, an important difference in the rates of amino acid uptake can be shown by comparing alanine uptake by larvae and adults. Wright and Stephens (1978) report that a mussel weighing 5 g (wet wt minus shell) can accumulate amino acids from a micromolar solution at a rate of 2 μmoles/h at 20°C. Assuming 20% of the mussel's weight is dry organic weight, an adult could take up alanine at a rate of 178 μg alanine (g dry wt)$^{-1}$ h^{-1}. In this work, larvae of *M. edulis* were observed

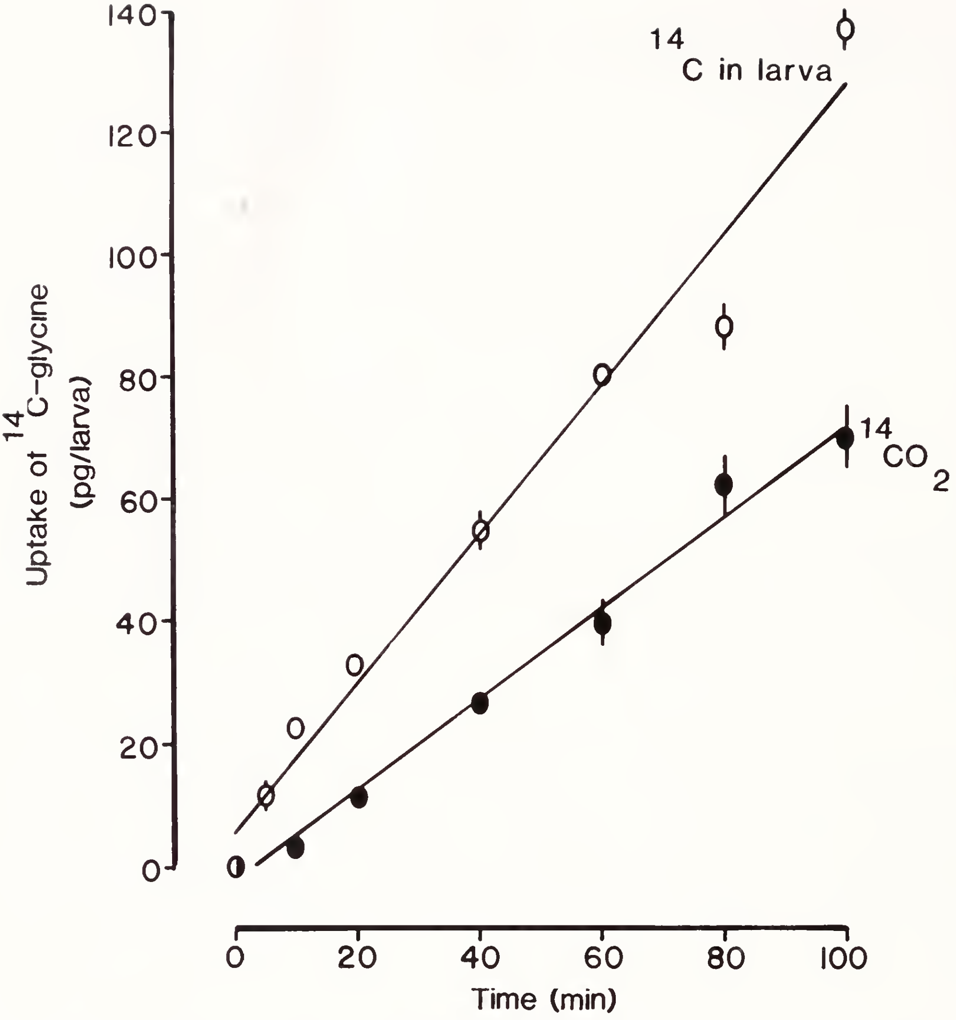

FIGURE 3. Time course of ^{14}C-glycine uptake and oxidation by *C. gigas* veligers. ^{14}C-glycine concentration = 0.69 μM. Temperature = 25°C. Salinity = 32‰. Open circles represent ^{14}C recovered in larvae. Closed circles represent $^{14}CO_2$ produced by larvae. All data points are mean ± S.E. for n = 3. Where no bar errors are shown on a data point, the bars lie within the graphical presentation of the point.

Regression equations

Treatment	*n*	*a*	*b* ± *S.E.*	r^2
Uptake by larva	24	5.48	1.22 ± 0.05	0.97
$^{14}CO_2$ production	21	−2.32	0.74 ± 0.03	0.96

to accumulate alanine from a micromolar solution at a rate of 180 pg larva^{-1} h^{-1} at 15°C. Crisp (1976) recalculated the dry organic weight of a *M. edulis* larvae as 0.2 μg from Zeuthen's (1947) values of nitrogen content. Taking this figure, a larva would accumulate 0.9 mg alanine (g dry wt)$^{-1}$ h^{-1} at 15°C. At 20°C, corresponding

Table IV

The metabolism of ^{14}*C-glycine by* C. gigas *veligers*

Time		Metabolic fraction	
		(pg ^{14}C-gly/larva)	(% of total)
10 min	Total uptake	25.6	
	Lipid	0.1	0.004
	Protein	2.9	11.3
	CO_2	3.2	12.5
	TCA soluble	19.4	75.8
100 min	Total uptake	206.9	
	Lipid	1.1	0.5
	Protein	33.2	16.0
	CO_2	69.8	33.7
	TCA soluble	102.8	49.7

to Wright and Stephens' experiment, the rate would be higher. For example, assuming a Q_{10} of 2, a larva could take up 1272 μg alanine (g dry wt)$^{-1}$ h^{-1} at 20°C compared to a value of 178 μg alanine given above for the adult. Thus, on a relative weight basis, the larva is taking up alanine at a rate approximately one order of magnitude faster than that of the adult. Presumably this difference is a reflection of the greater absorption surface area to volume ratio of the larva.

Rates of metabolism of absorbed amino acids

Bamford and McCrea (1975) and Stewart and Bamford (1975) showed that in the adult cockle *Cerastoderma edule* and the clam *Mya arenaria* 95% of the labeled amino acid taken up remained unmetabolized after one hour of exposure, and in some cases it took up to 24 h for the major portion to be metabolized (*e.g.*, Stewart, 1977). The data presented here show that in larvae, the time scale is much shorter. For instance, Table III shows that *C. gigas* pediveligers metabolize at least 61% of the absorbed ^{14}C-glycine in just 10 min. Following 100 min exposure, only 12.8% of the ^{14}C-labeled glycine remained in the TCA soluble fraction, 87.2% of the ^{14}C appeared as lipid, protein, and $^{14}CO_2$.

The above studies show, not only that bivalve larvae have a transport system operating at a high affinity for absorbing amino acids at environmentally realistic concentrations, but also that, in comparison with adults, larvae have a more rapid weight-specific uptake, and a faster rate of metabolising these substrates.

Contribution of amino acid uptake to energy and growth requirements

Johannes *et al.* (1969) drew attention to the critical difference between an influx of radiolabeled substrate and a net entry of that substrate. Although the question of net flux has not been addressed in this study, Manahan *et al.* (1982) showed that adult mussels *M. edulis* are capable of net uptake of amino acids from concentrations as low as 38 n*M*. Recent work has shown a comparable picture with respect to net influx of amino acids in bacteria-free sea urchin larvae (Manahan *et al.*, 1983) and *C. gigas* larvae (unpublished data).

The uptake of dissolved organic nutrients by an organism has customarily been compared with it's respiration rate (*e.g.*, Stephens, 1964; Shick, 1975; Wright and

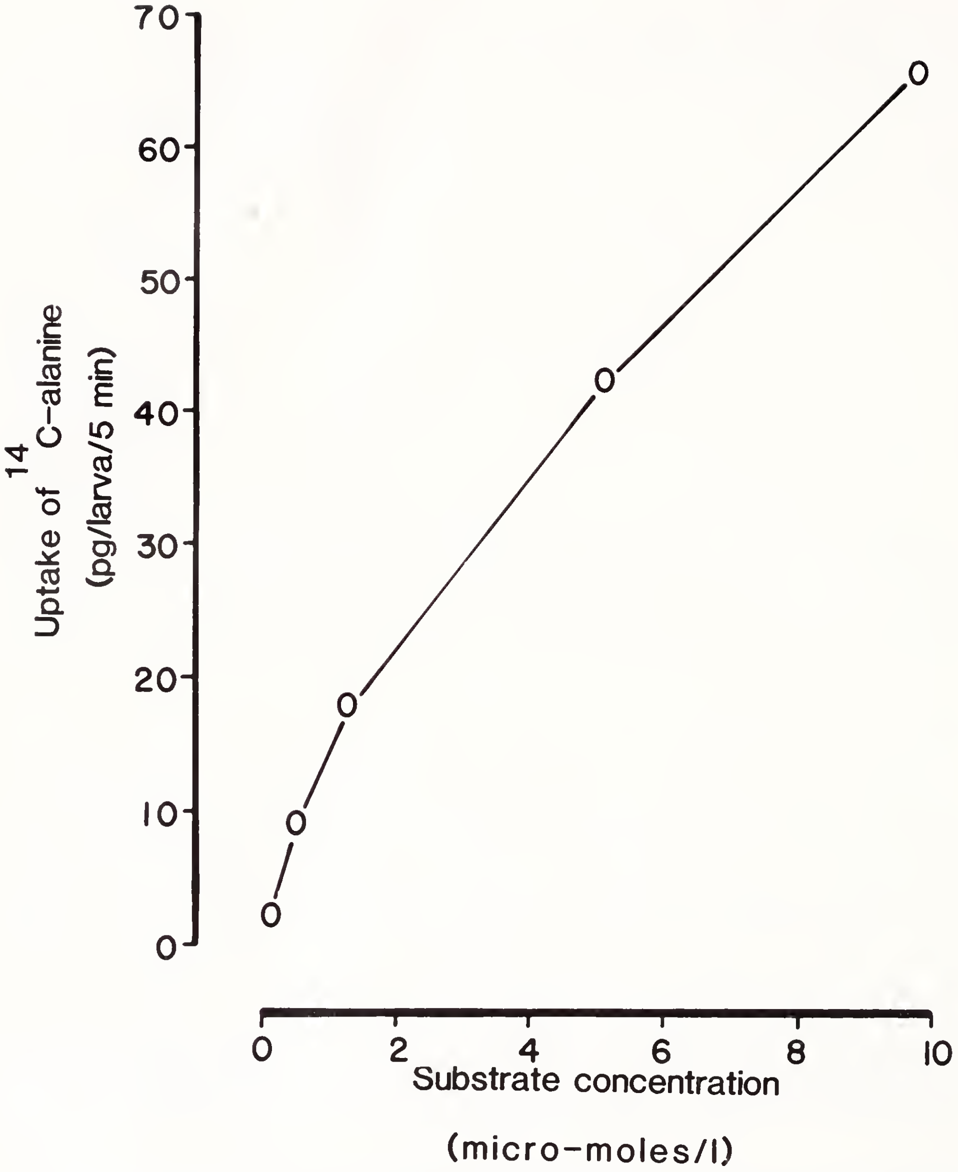

FIGURE 4. The uptake of ^{14}C-alanine by *M. edulis* veligers at substrate concentrations ranging from 0.1 μM to 10 μM. Temperature = 15°C. Salinity = 32‰. Data points are mean ± S.E. for n = 3. Where no bar errors are shown on a data point, the bars lie within the graphical presentation of the point.

Stephens, 1978). The rationale of this approach is that the dissolved organic substrates taken up can either themselves be oxidized, or can spare the animal's own energy reserves in providing energy for maintenance. This may be approximately true for full size adults without current reproductive expenditure, but it is manifestly inappropriate for growing larvae. The net growth efficiency, defined as rate of increase of biomass (or production, P) as a percentage of total energy requirements

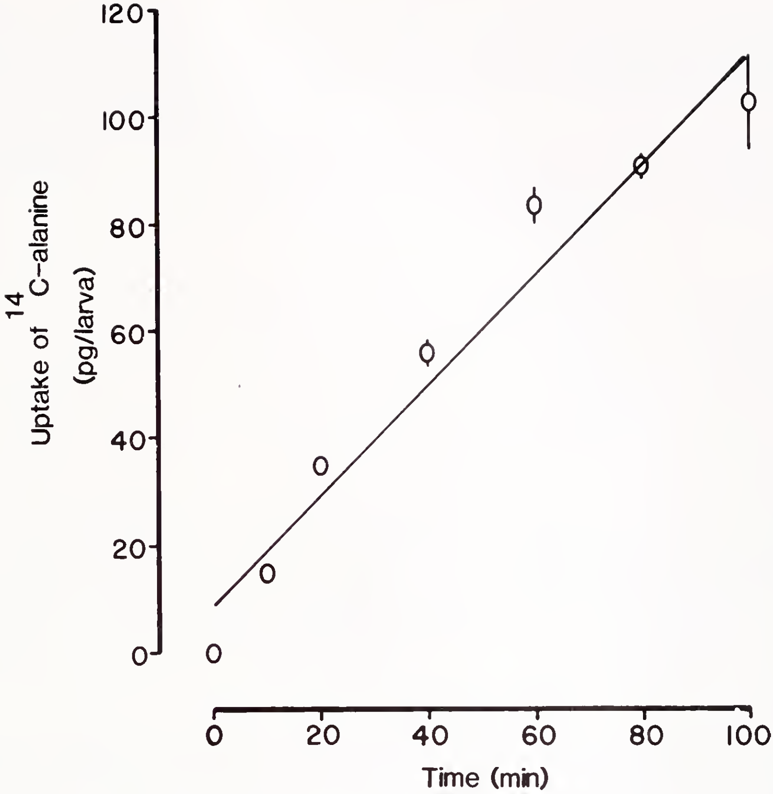

FIGURE 5. Time course of ^{14}C-alanine uptake by *M. edulis* veligers. ^{14}C-alanine concentration = 0.41 μM. Temperature = 15°C. Salinity = 32‰. Data points are mean ± S.E. for n = 3. Where no bar errors are shown on a data point, the bars lie within the graphical presentation of the point.

(P + respiration, R), is high in marine invertebrate larvae, usually about 70% (Holland, 1978), whereas in a hypothetical non-growing non-reproducing adult it would be zero. Therefore it is important to consider the energy cost of growth as well as respiration in larvae although not, of course, the cost of reproduction.

Data on cost of growth in developing pelagic larvae are scanty, and none exist for *C. gigas* veligers. Fortunately, comprehensive measurements have been made on the growth of the European oyster *Ostrea edulis* (Holland and Spencer, 1973), over the whole larval period including the late veliger stage. The latter stage corresponds in size, morphology, and linear growth rate with the 300 μm shell length *C. gigas* veligers used in this study and is therefore a very appropriate model. Table VI gives the rates of increase for each major biochemical component (Column I) for the 297 μ veliger of *O. edulis*. The total energy increase in the larva is computed as follows. Each component's (Column IV) daily percentage increase is multiplied by it's respective weight per larva (Column II) and by it's caloric content using values given by Crisp (1971). The results are the daily energy increases for each component, listed in Column V. These contributions are added to obtain the total figure of 22.86 × 10^{-4} cal larva^{-1} day^{-1}. To obtain the total energy requirements, the energy loss in respiration by *C. gigas* larvae must be added. The latter was obtained by multiplying the experimental value of 10 nl O_2 larva^{-1} h^{-1} (=240 nl

TABLE V

The metabolism of ^{14}C-alanine by M. edulis *veligers*

Time		Metabolic fraction (pg ^{14}C-ala/larva)	Metabolic fraction (% of total)
10 min	Total uptake	15.3	
	Lipid	0.2	1.3
	Protein	2.1	13.7
	CO_2	—	—
	TCA soluble	13.0	85.0
100 min	Total uptake	102.7	
	Lipid	1.0	1.0
	Protein	21.1	20.5
	CO_2	—	—
	TCA soluble	80.6	78.5

larva^{-1} day^{-1}) by the accepted oxycalorific equivalent of 4.8 Kcal/l O_2. Thus, the total energy requirement arrived at for *C. gigas* is 34.38 × 10^{-4} cal larva^{-1} day^{-1}.

The energy contributed by uptake of dissolved amino acids will depend on the ambient concentrations to which larvae are exposed. These are likely to range from 0.6 μM for surface waters to 6.0 μM for sediment waters (Henrichs and Farrington, 1979). The latter concentration may be relevant as larvae approach settlement. The rate of glycine uptake at these concentrations is 0.26 ng and 1.2 ng larva^{-1} h^{-1} (Fig. 1). Glycine has a molar heat of combustion of 233 Kcal/mole, giving 0.19 × 10^{-4} and 0.87 × 10^{-4} cal larva^{-1} day^{-1}, respectively. The uptake of glycine at these two concentrations would represent 0.6% to 2.8% of the total energy required by the larva. As a percentage of the energy respired (11.52 × 10^{-4} cal larva^{-1} day^{-1}), the figures would clearly be larger, viz. 1.6% to 7.5%.

The other important aspect of uptake of dissolved nutrients is their contribution of material for growth. In the *C. gigas* pediveliger the percentage of glycine taken

TABLE VI

Energy requirements of an oyster larva

I Component	II Wt/larva (μg)	III % in 12 day-old fed larva (297 μm)	IV Percentage increase/day	V Energy required (cal larva^{-1} day^{-1})
Protein	1.09	59%	14%	8.62 × 10^{-4}
Polysaccharide Free reducing substances RNA	0.20	11%	16%	1.31 × 10^{-4}
Phospholipid	0.13	7%	18%	2.21 × 10^{-4}
Neutral lipid	0.42	23%	27%	10.72 × 10^{-4}
Total organic	1.84	100%	15%	22.86 × 10^{-4}
Shell	4.99	—	12%	—
Total larva	6.83	—	14%	—
Energy for respiration				11.52 × 10^{-4}
				=34.38 × 10^{-4}

Compiled from Holland and Spencer, 1973.

up which is incorporated into protein is 39% and 47% following a 10 min and 100 min exposure, respectively. Apart from loss as $^{14}CO_2$, much of the remaining ^{14}C-label may also be eventually utilized in tissue synthesis. A conservative estimate of 50% contribution to protein synthesis therefore seems reasonable. Hence, at the uptake rate from 0.6 μM and 6.0 μM glycine, the amount of amino acid going into protein would be 3.1 ng and 14.4 ng larva^{-1} day^{-1}. Comparing this with the estimated daily rate of protein synthesis (Table VI) of 1.09 μg multiplied by 14% = 152 ng larva^{-1} day^{-1}, the percentage contribution at the two concentrations tested would be 2.0% and 9.5%.

It will be noted that the contribution of absorbed glycine to total energy is distinctly less than that to growth as measured in terms of protein synthesis. This is to be expected since glycine has a very low caloric content (3.1 Kcal/g) compared with the main energy stores of protein (5.65 Kcal/g) and lipid (9.45 Kcal/g) characteristic of bivalve and other marine invertebrate larvae (Holland, 1978). Although the estimated contribution from amino acids at naturally occurring concentrations appears small, sea water also contains other organic compounds which are available for uptake (Williams, 1975; Wangersky, 1978). Marine invertebrates are capable of taking up these compounds from micromolar concentrations, and the evidence in favor of a significant role for this alternative nutritional pathway is now considerable (see review by Stephens, 1981). Furthermore, during periods when particulate food is scarce, this supplementary mode of nutrition may be vital for pelagic larvae which are frequently on critical energy budgets (Crisp, 1974).

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STUDIES ON THE REPRODUCTION OF THREE SPECIES OF *PEROPHORA* (ASCIDIACEA)*

HIDEO MUKAI,[1] HIROMICHI KOYAMA[2] AND HIROSHI WATANABE[2]

[1] *Department of Biology, Faculty of Education, Gunma University, Maebashi, Gunma 371, Japan, and*
[2]*Shimoda Marine Research Center, University of Tsukuba, Shimoda, Shizuoka 415, Japan*

ABSTRACT

The breeding season is summer for *Perophora formosana* and *P. sagamiensis*, and winter for *P. japonica*. In all species, the gonads lie on the left side within the first intestinal loop. Eggs at the bottom of the ovary mature first. Mature eggs are ovulated into the postero-dorsal corner of the right peribranchial cavity, where they gain access to the brood pouch. In *P. sagamiensis*, the branchial basket and digestive tract of a zooid disintegrate at the onset of brooding, and after sexual reproduction the whole zooid is resorbed. Very often, such degenerating zooids are replaced by new zooids budded from the basal stolon.

A new mode of asexual reproduction, termed "terminal budding," was found in *P. japonica*. The distal end of a stolon rises up off the substratum and develops into a discoidal stellate reproductive body which is gorged with granular amoebocytes. This terminal bud is separated from the colony, then is carried away by water currents and adheres to a new substratum, on which it elongates stolons to found a new colony. Terminal budding can coexist with ordinary subterminal budding in the same stolon, but it is suppressed by sexual reproduction.

P. formosana is considered more primitive than the other two species.

INTRODUCTION

The genus *Perophora* consists of colonial ascidians, familiar to most marine biologists. In the colony, all the zooids are connected basally by branched creeping stolons from which they have arisen as buds. Generally, budding occurs successively at a short distance from the tip of a growing stolon. The stolon contains a vertical mesenchymatous septum which divides the lumen into two channels. This septum disappears near the distal end of the stolon allowing continuity of blood flow between the right and left channels. *Ecteinascidia* is another colonial ascidian closely allied to *Perophora*. These two genera constitute the family Perophoridae (Van Name, 1945; Berrill, 1950).

Asexual reproduction of *Perophora* has been the subject of many studies over a long period of time. The course of blastogenesis was studied by Kowalevsky (1874), Ritter (1896), Lefèvre (1898), Brien and Brien-Gavage (1927), and Deviney (1934). Freeman (1965) showed experimentally that the lymphocyte, a type of blood cell, participates in the initiation of blastogenesis. The behavior of isolated stolon pieces or zooid-stolon preparations under various external conditions has been observed by Huxley (1921), Goldin (1948), Barth and Barth (1966), and Fukumoto (1971). Sexual reproduction of *Perophora* has received comparatively little attention. All species of *Perophora* are ovoviviparous; embryogenesis takes place in a brood pouch.

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The activities and structure of the free-swimming larva were studied by Grave and McCosh (1923), and some aspects of metamorphosis by Oka (1960) and Terakado (1967). Recently, Fukumoto (1981) described the ultrastructural features of the sperm. As to *Ecteinascidia*, the origin of gonads and sex cells was studied by Simkins (1924) and blastogenesis by Plough and Jones (1939).

We have been rearing three species of *Perophora* for these several years, and have studied their sexual and asexual reproduction revealing some new facts. Comparative morphology of their gonads and brood pouches, and two new modes of asexual reproduction, called "terminal" and "replacement" budding, constitute the major contents of this paper.

MATERIALS AND METHODS

The following three Japanese species of *Perophora* were used: *P. formosana* (Oka), *P. japonica* Oka, and *P. sagamiensis* Tokioka. For taxonomical remarks of these species, see Tokioka (1953).

Some colonies of *P. formosana* were originally collected at Shimoda (Shizuoka Prefecture), those of *P. japonica* in the vicinity of the Asamushi Marine Biological Station (Aomori Prefecture), and those of *P. sagamiensis* in the vicinity of the Seto Marine Biological Laboratory (Wakayama Prefecture). They were then reared in a small bay to which the Shimoda Marine Research Center is facing. All colonies were grown on glass plates which were set in boxes hung below the sea's surface. When necessary, the plates with the colonies attached were brought into the laboratory, and were observed under a stereomicroscope.

Observations on living material were supplemented with histological study. For this purpose, pieces of colonies were fixed in Bouin's fixative made in sea water. They were dehydrated through a graded series of ethanols; and paraffin sections, 7 μm thick, were stained with Delafield's hematoxylin and eosin.

RESULTS

Sexual reproduction: external observations

The breeding season of *P. formosana* and *P. sagamiensis* is summer, with the highest activity during July and August; that of *P. japonica* is winter, with the peak period during December and January.

Perophora is hermaphroditic like other ascidians, and is more or less protandrous. Both the testis and the ovary lie within the first intestinal loop, in the mantle on the left side of the body. The sperm duct accompanies the rectum and opens into the cloacal cavity shortly anterior to the anus. Embryogenesis takes place within the brood pouch located in the right peribranchial cavity. The oviduct leading to the brood pouch is very hard to demonstrate in live or fixed total specimens. Anatomical details of the gonads and the brood pouch are different from species to species.

In *P. formosana*, the testis is a large oval mass located near the ventral corner of the intestinal loop. The ovary is located on the sperm duct at some distance from the testis (Fig. 1A). The brood pouch is a tubular organ, extending dorso-ventrally in the posterior portion of the right peribranchial cavity (Fig. 1B). The lumen of the brood pouch communicates with the peribranchial cavity through the ventral or distal opening. As ovulation proceeds, the brood pouch grows in length distalward. Ovulation continues in a chronological order, so that embryos are naturally arranged in a series with the earliest in developmental stage at the proximal end of the pouch

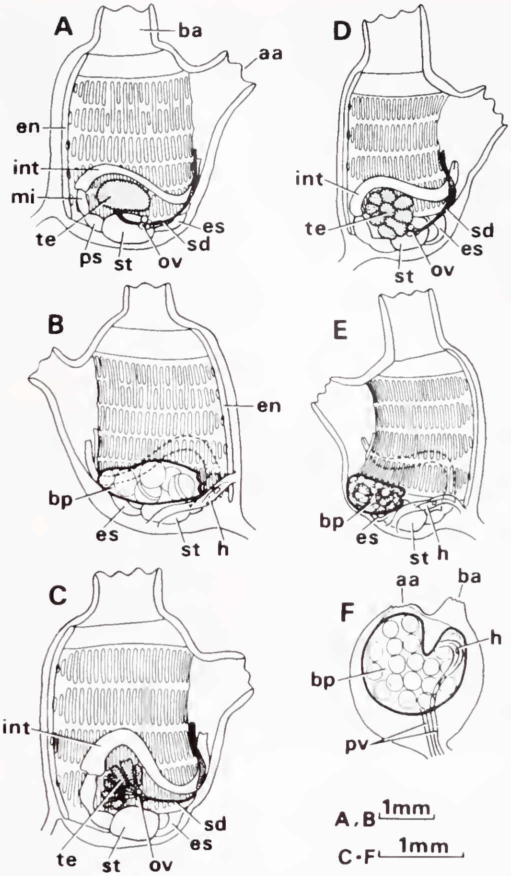
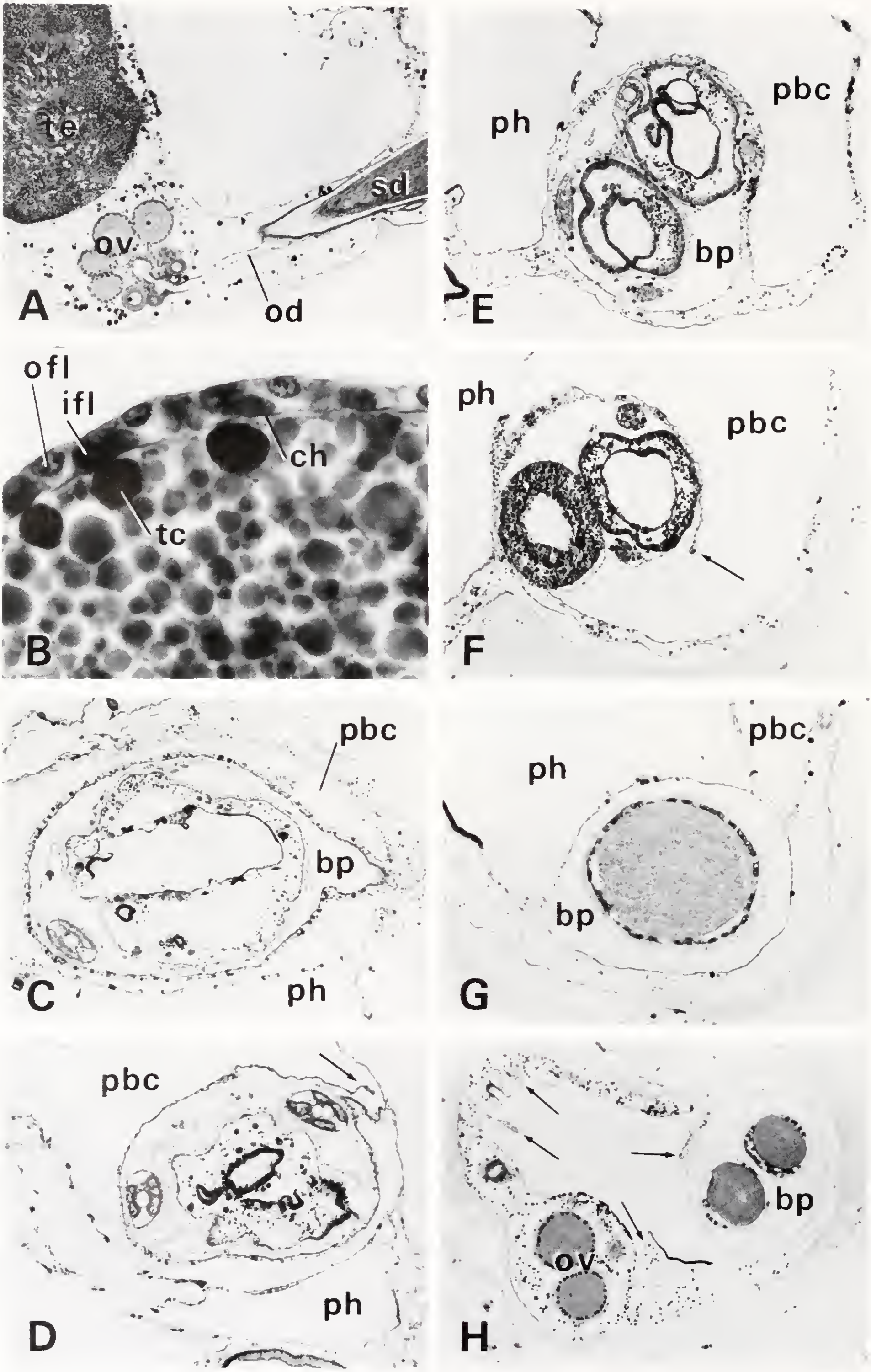


FIGURE 1. External views of zooids, showing gonads and brood pouch. All figures were drawn from living specimens with the aid of a camera lucida. A: *P. formosana*, from left side. B: *P. formosana*, from right side. C: *P. japonica*, from left side. D: *P. sagamiensis*, from left side. E: *P. sagamiensis*, from right side. F: A sac-like zooid of *P. sagamiensis*, crowded with embryos after the resorption of the viscera, from right side. aa, atrial aperture; ba, branchial aperture; bp, brood pouch with embryos; en, endostyle; es, esophagus; h, heart; int, intestine; mi, mid-intestine; od, oviduct; ov, ovary (immature); ps, post-stomach; pv, peduncular vessels; sd, sperm duct; st, stomach; te, testis (fully grown).

and the most advanced at the distal end. Less than ten embryos develop within the pouch at one time. As soon as the tadpole stage is completed, the embryos leave the brood pouch and then the mother zooid through its atrial aperture as free larvae.

In *P. Japonica*, the testis is divided into 6–10 wedge-shaped follicles which are arranged fanwise. Small sperm ducts issuing from respective follicles are united at the rivet of the testicular fan to form a common sperm duct. The ovary is located at the starting point of the common sperm duct (Fig 1C). The brood pouch of this species is quite similar in appearance to that of *P. formosana*. More than 10 embryos are found at a time in a zooid. Nearly mature embryos are often pushed out of the brood pouch into the peribranchial cavity. They soon hatch and escape from the zooid through its atrial aperture as free-swimming larvae.

In *P. sagamiensis*, the testis is subdivided into several irregular lobes Tokioka (1953) reported in the original description of this species that the testis is a large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oval mass. In all probability, this is an error. Our material agrees with Tokioka's description of the zooid except for the testis. As in *P. japonica*, the ovary is located close to the testis (Fig. 1D). The brood pouch initially appears as a small sac-like organ near the postero-dorsal corner of the right peribranchial cavity as in *P. formosana* and *P. japonica;* but unlike these species, the brood pouch of *P. sagamiensis* is situated below the branchial basket or pharynx (Fig. E). A network of blood lacunae is conspicuous in the wall of the brood pouch. At about the time when the brood pouch contains a few embryos, the mother zooid closes its branchial and atrial apertures, the former permanently and the latter temporarily, until the liberation of larvae. Within the zooid, the branchial basket and digestive tract disintegrate and are fully resorbed; concomitantly, the brood pouch augments more and more occupying the cavity formed by the disappearance of the branchial basket. Thus, after ovulation of all eggs, the zooid becomes a closed sac filled with growing embryos, about 20 in number (Fig. 1F). The heart continues to beat even in this condition. When embryos develop into mature tadpoles, they swim out through the now slightly opened atrial aperture. Following the liberation of all larvae, the empty sac of the mother zooid shrinks and is eventually resorbed into the basal stolon.

Sexual reproduction: histological observations

The zooids of *P. formosana* and *P. japonica* are protandrous. Mature sperm appear in the testis somewhat prior to the onset of ovulation. Subsequently, the testis continuously diminishes in size though it contains some sperm almost throughout the period of ovulation. On the other hand, each zooid of *P. sagamiensis* is definitely protandrous (Fig. 2A). The testis sheds all the sperm prior to ovulation, or at least before the onset of the involution of the branchial basket. In all species, the sperm duct consists of cuboidal cells and is sparsely ciliated, except near the distal end where it is heavily ciliated.

The following description of the ovary is common to all species studied. A young ovary is essentially an oval vesicle consisting of squamous epithelial cells and small previtellogenic oocytes. Towards the blind end of the ovary, oocytes are seen to become successively larger (Fig. 2A); this fact was illustrated with *Perophora listeri* by Kowalevsky (1874). These small oocytes are surrounded by a single layer of primary follicular cells and are embedded in the ovarian wall. As they grow larger, they protrude outward accompanied by individual short follicle stalks which are continuations of the ovarian wall. A mature oocyte is surrounded by a pair of follicular layers, the outer and the inner, both consisting of squamous or somewhat cuboidal cells, and a thin non-cellular layer known as the chorion (Fig. 2B). Beneath the chorion, the so-called test cells are scattered; their cytoplasm is filled with intensely basophilic globules. The oocyte is polylecithal and contains numerous eosinophilic yolk granules. The oviduct consists of a single layer of squamous epithelial cells and is sparsely ciliated. The oviduct, which lies more superficially than the

FIGURE 2. Sections of sexual reproductive organs. A: *P. sagamiensis*, section through an immature ovary and the sperm duct. ×90. B: *P. japonica*, part of a mature egg. ×1000. C: *P. formosana*, cross section through a proximal part of the brood pouch. ×70. D: *P. formosana*, cross section through a distal part of the brood pouch. Arrow indicates the free edge of the wall of brood pouch. ×70. E: *P. japonica*, cross section through a proximal part of the brood pouch. ×70. F: *P. japonica*, cross section through a distal part of the brood pouch. Arrow indicates the free edge of the wall of brood pouch. ×70. G: *P. sagamiensis*, cross section of an early brood pouch. ×100. H: *P. sagamiensis*, a zooid at an early stage of brooding. Arrows indicate remnants of the branchial basket. ×40.bp, brood pouch; ch, chorion; ifl, inner follicular layer; od, oviduct; ofl, outer follicular layer; ov, ovary; pbc, peribranchial cavity; ph, pharynx; sd, sperm duct; tc, test cell; te, testis.

sperm duct, accompanies the proximal half of the sperm duct but fails to follow its distal curve; the oviduct extends to and beyond the middorsal line to open in a small lobulated projection at the proximal end of the brood pouch. Upon ovulation, the outer follicle is cast off in the ovary and the egg is discharged into the brood pouch. Probably, fertilization takes place in the brood pouch, where the newly ovulated egg is heavily surrounded by sperm. The fertilized egg undergoes cleavage and embryogenesis within the expanded chorion, over which stretched inner follicular cells are attached.

In all species, the brood pouch is a tubular organ lined by the peribranchial epithelium, whose cells are little specialized and are quite similar in appearance to those of other parts. In *P. formosana*, the brood pouch is wholly attached to the mantle. In cross section, the brood pouch appears as a perfect tube, except for a short distance near the distal end where it is broken or discontinuous at a point toward the outer margin (Figs. 2C, D; 3A, B). In *P. japonica*, the brood pouch occupies the immediate posterior end of the peribranchial cavity; thus, a basal part of the branchial wall participates in its formation. Therefore, the brood pouch of this species is situated somewhat posteriorly as compared with that of *P. formosana*. As in *P. formosana*, the brood pouch of *P. japonica* is a perfect tube in its proximal portion; but near the distal end it is discontinuous at a point toward the mantle (Figs. 2E, F; 3C, D). The brood pouch of *P. sagamiensis* initially develops more posteriorly than that of *P. japonica*. It lies below the peribranchial cavity (Figs. 2G, 3E). Around the distal opening of the brood pouch leading to the peribranchial cavity, the epithelial cells are thickened and are able to close the opening (Fig. 3F). These observations, coupled with the external observations described before, clearly show that in *P. formosana* the brood pouch is formed by the growth of a partition wall within the original peribranchial cavity; while in *P. sagamiensis* the brood pouch is formed by an evagination from the floor of the peribranchial cavity into the posterior part of the mantle. The brood pouch of *P. japonica* seems to be formed by a combination of these two ways, with the growth of a partition wall playing the major part.

As described earlier, in *P. sagamiensis* the branchial basket and digestive tract are resorbed soon after the initiation of brooding, and the brood pouch expands, filling up the space formed by their resorption (Fig. 2H). It should be noted that the brain ganglion remains intact throughout the period of brooding.

Ordinary budding or subterminal budding

In every species of *Perophora*, a budding site is located a short distance from the tip of a growing stolon, where a bud arises and develops into a zooid. This ordinary type of budding is here termed subterminal budding. As the stolen elongates, it produces successively new buds. Therefore, so far as the colony grows by subterminal budding, zooids are arranged on the stolon in order of age with the youngest next to the growing tip. Each zooid is situated with the ventral side (*i.e.*, the side of endostyle) directed toward the distal end of the stolon.

The course of blastogenesis in subterminal budding has been well documented by earlier workers (Lefèvre, 1898; Brien and Brien-Gavage, 1927; Deviney, 1934). Consequently, we are not especially concerned with this subject in the present study. Only a few words will suffice. A bud primordium consists of a double vesicle. The outer vesicle is derived from the epidermal wall of the stolen and gives rise to the epidermis of the zooid. The inner vesicle is derived from the septum of the stolon and gives rise to all adult tissues and organs except the epidermis. For the formation

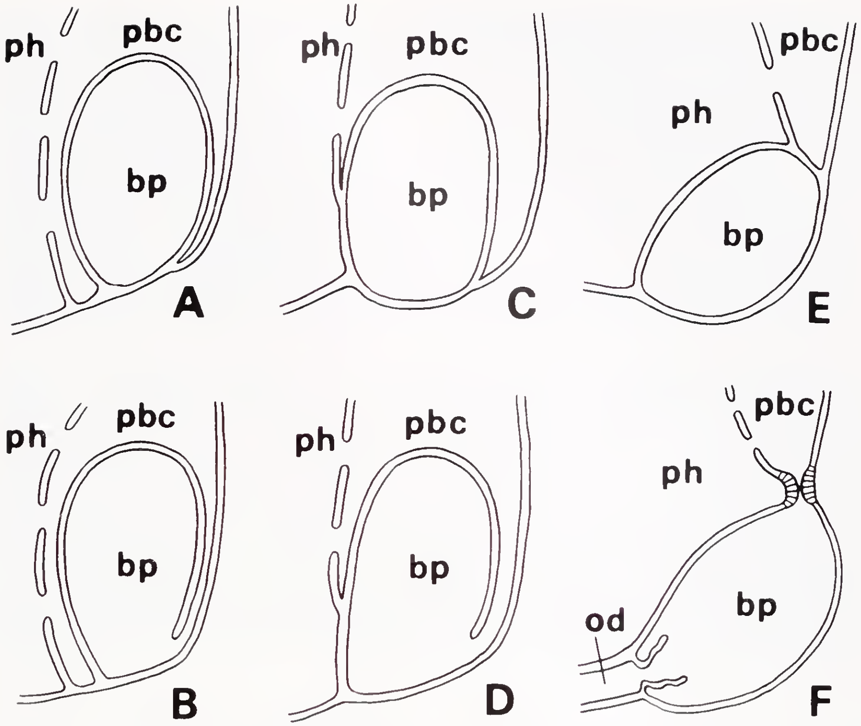

FIGURE 3. Diagrammatic representation of the brood pouch. A: *P. formosana*, cross section through a proximal part of the brood pouch. B: *P. formosana*, cross section through a distal part of the brood pouch. C: *P. japonica*, cross section through a proximal part of the brood pouch. D: *P. japonica*, cross section through a distal part of the brood pouch. E: *P. sagamiensis*, cross section of an early brood pouch. F: *P. sagamiensis*, longitudinal section of an early brood pouch. bp, brood pouch; od, oviduct; pbc, peribranchial cavity; ph, pharynx.

of the inner vesicle and its subsequent organogenesis, lymphocytes in the circulating blood also may be employed (Deviney, 1934; Freeman, 1964). This mode of blastogenesis is generally known as stolonial budding.

The colony is extended by terminal growth and lateral branching. At least in the three species presently examined, the elongation of stolon occurs only near its tip, in the portion distal to the youngest bud. The branching of stolon or the formation of new stolons occurs most vigorously near the distal end of a growing stolon and around the bases of developing buds. In *P. formosana*, when the growing tip of a stolon encounters another stolon of the same colony, the former usually continues to grow crossing over the latter. However, in *P. japonica* and *P. sagamiensis*, stolons of the same colony easily anastomose and fuse together, thus forming a network of stolons.

Dormant stolons

In Shimoda, *P. formosana* undergoes hibernates, as reported briefly by Fukumoto (1971) with *Perophora orientalis*, which is homospecific with *P. formosana* (*cf.* Tokioka, 1953). Every year, all the zooids and buds regressed in late autumn (November), and only stolons survived the winter months (Fig. 4A). The overwin-

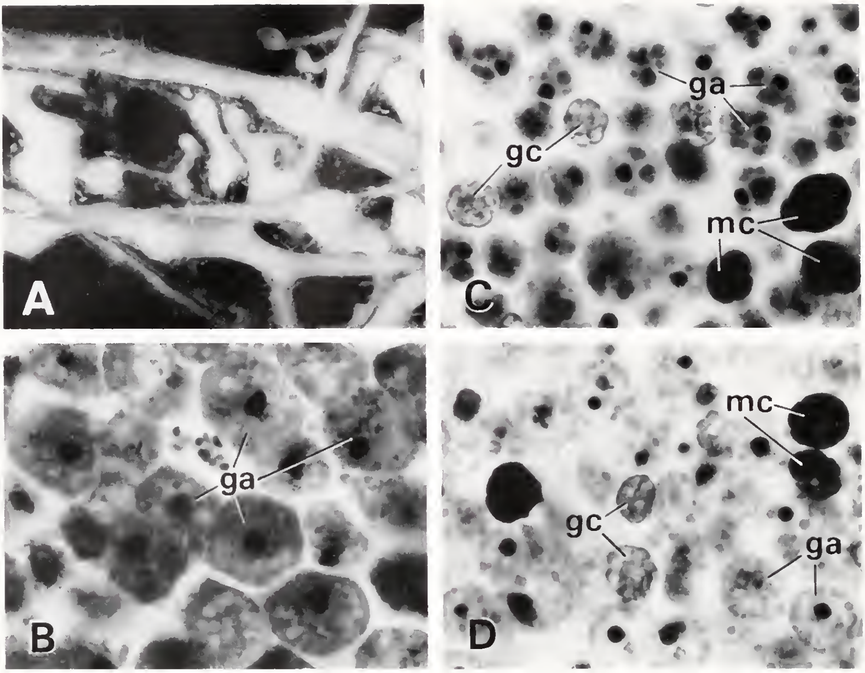

FIGURE 4. Dormant stolons. A: *P. formosana,* external appearance. ×12. B: *P. formosana,* congested blood cells. ×1000. C: *P. japonica,* congested blood cells. ×1000. C: *P. sagamiensis,* congested blood cells. ×1000. ga, granular amoebocytes; gc, green cells; mc, morula cells.

tering stolons were opaque and yellowish, owing to the accumulation of blood cells. Such packed opaque stolons, in which development is inhibited until the environment becomes more favorable, are generally termed dormant stolons in this paper. The dormant stolons were often constricted into bulges, where the tunic grew thicker and harder than usual. The bulges might be isolated, singly or in connection of several bulges, from other stolons by disintegration of the latter, though this was not an inevitable process.

The overwintered dormant stolons of *P. formosana* produced new blastozooids from late April to early May. The new blastozooids were often budded directly from old stolons; but the bulged portions of stolons first elongated new stolonic outgrowths, which then produced subterminal buds.

The colonies of *P. sagamiensis* showed a tendency to hibernate, though their behavior during winter differed from year to year. In some years, almost all zooids regressed in winter, and stolons survived the winter and regenerated zooids in late spring as in *P. formosana.* However, in some other years many zooids escaped regression and survived the winter.

Generally, *P. japonica* colonies continued to grow with functional zooids throughout the year. However, in midsummer (August) their zooids occasionally regressed to disappear from limited areas, leaving dormant stolons. Such dormant stolons sent out slender new stolons within a few weeks or so. Thus, this species shows a slight tendency of estivation instead of hibernation, at least in Shimoda.

Blood cells of *Perophora viridis* have been divided into several morphologically distinct classes (George, 1926; Freeman, 1964; Overton, 1966). In histological preparations, the dormant stolons of all species studied are filled with blood cells. The most abundant cells are spherical and contain coarse eosinophilic granules in the cytoplasm. These cells function as trophocytes and can be identified as granular amoebocytes in the terminology of the previous authors, though their size and appearance are somewhat variable with the species (Fig. 4B–D). In addition to the granular amoebocytes, berry-like cells consisting of bright yellowish-green bodies, the green cells, are relatively common in the dormant stolons of all species. Morula cells, which are intensely stained with hematoxylin and eosin, are found in the dormant stolons of *P. japonica* and *P. sagamiensis;* but this cell class is absent from the blood of *P. formosana.*

Terminal budding in Perophora japonica

It was found that in *P. japonica* the distal tip of a growing stolon turns into a kind of asexual reproductive body, which is eventually severed from the stolon and drifts away to found a new colony. This type of asexual reproduction has never been reported before, and is termed "terminal budding" in this paper.

Terminal buds at various stages of formation are shown in Figure 5A. In a "normal" stolon, the growing tip is fully attached to the substratum by the lower side, and the septum usually terminates in a flattened vesicle (Fig. 6A). At the start of terminal budding, a distal portion of the stolon rises up off the glass plate, losing contact with the substratum, and grows into a hyperplastic bulge. Then, the terminal

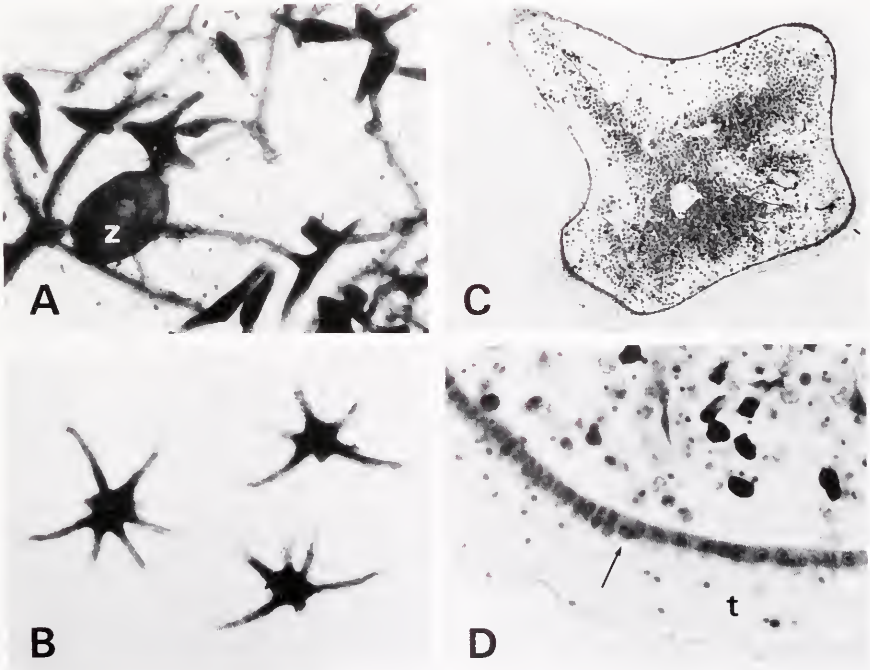

FIGURE 5. Terminal buds of *P. japonica.* A: Terminal buds at various stages of their formation. Extremely thin stolons are of hydrozoan colonies. z, zooid. ×8. B: Isolated terminal buds. ×8. C: Section of an early terminal bud. Blood cells, mostly granular amoebocytes, are accumulated in the lumen; septal walls are branched. ×70. D: Part of an early terminal bud, showing thickened epidermal cells and a mitotic figure (arrow). t, tunic. ×400.

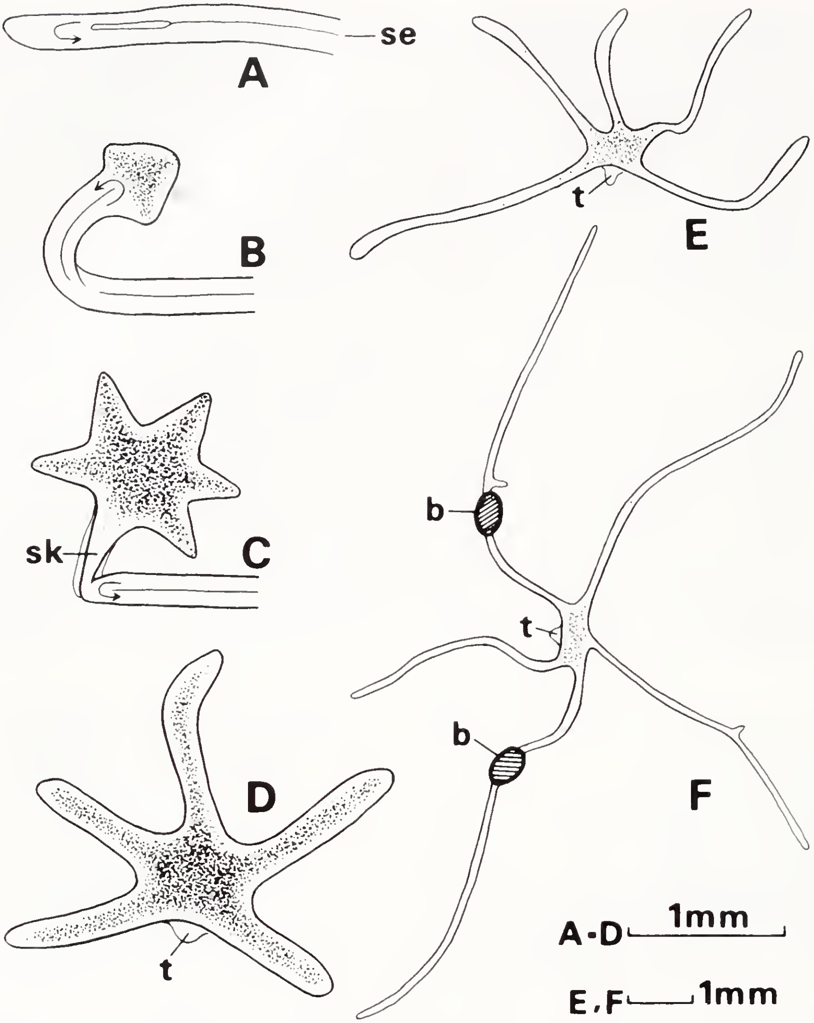

FIGURE 6. Diagrammatic representation of the formation and development of the terminal bud in *P. japonica.* A: Normal growing tip of a stolon. B: Early stage of terminal budding. C: Terminal bud before isolation. D: Terminal bud soon after isolation. E: One day after isolation. F: Two days after isolation. b, subterminal bud; se, septum; sk, stalk; t, tunic as a vestige of the stalk.

bulge, containing the terminal vesicle of the septum, expands fanwise (Fig. 6B). Subsequently, it develops epidermal projections, 3 to 6 in number, along its margin (Fig. 6C). These projections are stolon primordia. During these processes, the terminal bud becomes opaque by the accumulation of blood cells; and, the peduncular portion or stalk that connects the bud proper and the basal creeping stolon curves more and more upward until the bud stands straight or even until it turns upside down. The blood flow in the stalk ceases, and at last the bud becomes fully severed from the stolon. The isolated terminal bud is discoidal and stellate in outline (Figs. 5B, 6D); its stolons are now elongating. In the summer season (at about 25°C), it takes about 2 days from the initiation of swelling of the stolon tip to the isolation of it as a mature terminal bud. A stolon forms only one terminal bud at a time, yet it can produce many consecutively. Both terminal and subterminal budding can occur simultaneously in the same stolon.

In sections, the terminal bud consists of tunic, epidermis, septal walls, and blood; no sign of blastogenesis is found in it. The majority of the blood cells are granular amoebocytes, and some others are green cells and morula cells. The mesenchymatous septum is highly branched (Fig. 5C). The epidermis is thickened in the stolon primordia, where mitotic figures are common during their formation (Fig. 5D).

A free terminal bud may be carried away by water currents to some distance. Its surfaces are very sticky; therefore, it adheres to almost any solid substance that it may chance to encounter. After attaching to a substratum, the bud elongates its stolons rapidly, forming a new colony without zooids (Fig. 6E). Then, after the stolons have grown to considerable lengths, they develop buds which grow into zooids. The number of the initial buds is usually less than three in a colony; a stolon can produce only a single bud at a short distance from the tip (Fig. 6F). That is, the appearance of blastozooids in this colony is effected by ordinary subterminal budding. In running sea water at about 25°C, the initial blastozooids develop into vesicular buds in about 2 days after isolation of the terminal bud; and about 2 more days pass before they differentiate into functional zooids. In standing sea water, these processes proceed more slowly.

A slow irregular circulation of blood is seen in the stolons growing from the terminal bud, even before the appearance of the initial buds or blastozooids. This blood flow is clearly caused by the contraction of stolonic epidermis. At first, each stolon has only one channel. Later, as nutritive blood cells are consumed, the stolon acquires two channels, developing a septum in the lumen. In such a stolon, blood only flows still back and forth, with a 2–3 min cycle. Blood flow reversals are usually not synchronized in the stolons of the same colony. Even when the hearts of the initial zooids start beating, blood flow in opposite directions between two channels as in normal stolons is limited to short ranges near the zooids. Somewhat similar circulation of blood which is effected by the contraction of epidermal cells has been observed by Huxley (1921) with isolated zooid-stolon preparations of *Perophora viridis*, especially during the later stages of zooid resorption.

In large colonies grown on glass plates, terminal budding took place vigorously throughout the non-breeding period of year. During the breeding season, gonads first appeared in some zooids located in the older parts of a colony. In such a colony, terminal budding continued only in those stolons which were distant from the zooids with mature gonads. As more and more zooids of the colony came to engage in sexual reproduction, terminal budding ceased in the colony. Even during the peak period of sexual reproduction of this species, under our culture conditions, some colonies remained showing little sexual activity. Such colonies continued forming terminal buds.

More information concerning the relation between terminal budding and sexual reproduction and that between terminal budding and colony age was obtained by the following observations. During August and September 1981, we observed two colonies founded by terminal buds. Thirty-five days after the settlement of the terminal buds, one colony had 27 functional zooids and 9 developing terminal buds, and the other colony had 10 functional zooids and 4 early terminal buds. In both colonies, no previous formation of terminal buds was noted. Similar observations were performed also during the early part of the breeding season, from October to November 1980, with seven colonies derived from terminal buds. Twenty-nine days after the settlement of the terminal buds, they were found bearing 57–90 functional zooids. Immature gonads were found in small numbers of zooids of only two colonies, but no terminal bud was found in any colony. After 43 days from the settlement, every colony had 240–420 functional zooids, a considerable number of which bore gonads. In four colonies, only 4–6 terminal buds were counted; and in three colonies no terminal bud was found. A week later, in all colonies many zooids had gonads and some of them carried even growing embryos; while none of the colonies bore a terminal bud. Similar results were obtained with colonies founded by metamorphosis of larvae. From October 1979 to January 1980, correspondingly to the peak period of sexual reproduction, we observed several such colonies. In this series, none of the colonies produced a terminal bud during the observation period of about 40 days. From these results, it is concluded that terminal budding is suppressed by sexual reproduction. Furthermore, these results suggest that juvenile colonies lack the capacity of terminal budding.

No evidence of terminal budding was found in *P. formosana* and *P. sagamiensis.*

Replacement budding in Perophora sagamiensis

As already described, the zooids of this species invariably degenerate after sexual reproduction. Often, but not always, such degenerated zooids are replaced by new blastozooids.

When a degenerating zooid appears as a minute knob, or sometimes after it has completely disappeared, a new bud appears on the stolon at the place where the original zooid has previously been located (Fig. 7). Generally, some accumulation of blood cells, largely granular amoebocytes, is found in the stolon near the bud. This bud develops into a functional zooid, with the same polarity as that of the original zooid. This type of budding is here termed "replacement budding" as distinct from subterminal budding; though, as judged by external observation, blastogenesis is alike in both types.

Replacement buds are very common during the sexual breeding season. They are also found during the remainder of the year, but are few in number probably

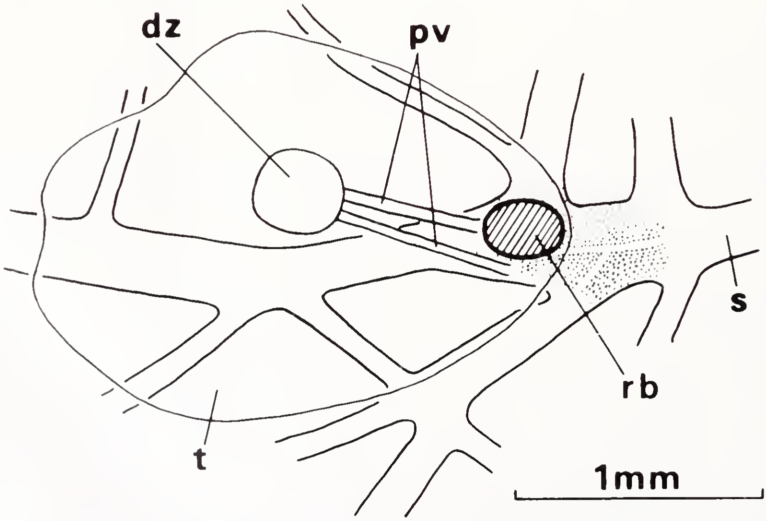

FIGURE 7. Replacement budding in *P. sagamiensis.* dz, degenerating zooid; pv, peduncular vessels of the degenerating zooid; rb, replacement bud; s, stolon; t, tunic ghost of the degenerating zooid.

because a relatively small number of zooids degenerate by physiological senescence in this period.

No sign of replacement budding is seen in *P. formosana* and *P. japonica.*

DISCUSSION

Perophora is an enterogonid ovoviviparous ascidian. The gonads are located on the left side, within the first intestinal loop. The sperm duct opens into the cloacal cavity; while the oviduct opens at the postero-dorsal corner of the right peribranchial cavity, discharging the eggs into the brood pouch consisting of the peribranchial epithelium. These features are also shared with *Ectanascidia* (see Simkins, 1924; Berrill, 1932).

The position of the brood pouch and the behavior of the mother zooid during brooding are different in different species. In *P. formosana* the entire brood pouch is attached to the mantle, but in *P. japonica* both the mantle and the branchial wall participate in the formation of the brood pouch. In these species, the zooid generally remains functional during and after sexual reproduction. On the other hand, in *P. sagamiensis* the brood pouch initially develops at a level posterior to the original peribranchial cavity. The branchial basket and digestive tract disintegrate soon after the onset of brooding, then the brood pouch expands into the space formerly occupied by the branchial basket. During the brooding period, the zooid remains as a sac with the heart and ganglion intact. After the completion of brooding, the zooid is wholly resorbed. A similar manner of brooding occurs in a pleurogonid colonial ascidian, *Botryllus primigenus* (Mukai, 1974, 1977).

The term "bud" or "budding" has been used in various senses in the literature of ascidian reproduction (*cf.* Berrill, 1935). A bud may be broadly defined as an asexually produced reproductive body, without reference to the presence or absence of the primordium of a blastozooid. Functionally, the buds defined above may be divided into three types: 1) buds which are concerned with the growth of the colony, 2) buds which are destined to found new colonies, and 3) buds whose function is primarily to survive adverse conditions. In the genus *Perophora,* these three types of buds are all found. The first type is represented by the subterminal bud, and the replacement bud may be considered a special form of this type. The terminal bud belongs to the second type, while the dormant stolon belongs to the third type.

In a more restricted sense, a bud may be defined as a rudimentary stage of a blastozooid. In this sense, the term budding is nearly equivalent to blastogenesis. In *Perophora,* only a single type of blastogenesis is known. That is, a blastozooid is always formed by the so-called stolonial budding. It is generally admitted that most colonial ascidians are given a single mode of blastogenesis. Only *Botryllus* and *Botrylloides* are known to be able to propagate by two different kinds of blastogenesis, *i.e.,* by palleal and vascular budding (Oka and Watanabe, 1957, 1959).

So far, only subterminal budding has been described in the natural growth of the colony in all species of *Perophora.* However, in *P. sagamiensis* new zooids are also formed by replacement budding, in addition to the ordinary subterminal budding. Consequently, in this species, the age-graded arrangement of zooids along the stolon which was once established by subterminal budding is put in disorder. Probably, the capacity of replacement budding may have developed in accordance with the habit of zooid degeneration following sexual reproduction.

In Shimoda, *P. formosana* undergoes typical hibernation. *P. sagamiensis* also displays hibernation to a lesser degree, while *P. japonica* shows a slight tendency of estivation. During hibernation or estivation, the colony survives in the form of dormant stolons filled with granular amoebocytes. Such stolons, of course, contain the mesenchymatous septum as a formative tissue, but blastogenesis is arrested until the environment becomes more favorable. The regression of zooids and subsequent formation of dormant stolons can also be induced experimentally under various adverse conditions in *Perophora listeri* (Brien and Brien-Gavage, 1927) and *P. viridis* (Huxley, 1921; Barth and Barth, 1966). Thus, the ability to produce dormant stolons appears to be potentially present in every species of *Perophora.* Somewhat similar dormant buds have long been known in the Clavelininae, in which new blastozooids develop from terminal ampullae of the so-called stolons or posterior extensions of the abdomens of individual zooids, after their isolation following the resorption of the zooids at the end of the sexual breeding season (*cf.* Berrill, 1950).

Our terminal bud was first described and illustrated as "a stellate structure" in the original description of *P. japonica* by Tokioka (1953). He considered its formation a diagnostic character of this species, but did not consider its function.

The terminal bud is similar to the dormant stolon. It is derived from a stolon following the accumulation of granular amoebocytes and in the absence of a zooid primordium. However, the dormant stolon is formed under unfavorable environmental conditions, and has a remarkable resting ability. By contrast, the terminal bud is formed only in an actively growing stolon. No evidence of arrested development of the terminal bud has been obtained. Terminal budding occurs concomitantly with subterminal budding, but is clearly suppressed by sexual reproduction. Further study is needed to know the regulatory mechanisms among these different modes of reproduction.

In the asexual reproduction of ascidians, only a few examples of dispersal mechanisms are known. Probably, the terminal bud of *Perophora japonica* is best compared with the planktonic-type bud of *Polyzoa vesiculiphora,* a polystyelid ascidian. In this species, two main types of palleal buds, stolonic and planktonic, are formed. The planktonic-type bud is congested with granular amoebocytes, isolated from the mother zooid, and may be carried away by water currents to found a new colony elsewhere (Watanabe and Tokioka, 1972; Fujimoto and Watanabe, 1976a, b). However, the planktonic-type bud of *Polyzoa* and the terminal bud of *Perophora* differ significantly. The former is essentially a zooid primordium that grows directly into a functional zooid, developing new test vessels from its ventral epidermis. By contrast, the terminal bud first grows stolons, which then produce zooids.

Terminal budding is apparently peculiar to *P. japonica.* This species is said to be very closely allied to *P. viridis, P. listeri,* and *P. banyulensis* (Tokioka, 1953). For similarities of the latter three species, see also Van Name (1945) and Berrill (1950). It is uncertain whether terminal budding also occurs in these species.

P. formosana differs from *P. japonica* and *P. sagamiensis* in many points. In *P. formosana,* a young zooid may have four rows of stigmata, but a full-grown zooid has five rows of stigmata with the first and second rows imperfectly separated; while the zooids of *P. japonica* and *P. sagamiensis* have strictly four rows of stigmata. *P. formosana* has a non-lobulated massive testis and an ovary situated at a distance from the testis; its blood lacks morula cells. By contrast, both *P. japonica* and *P. sagamiensis* commonly have a lobulated testis and an ovary situated at the proximal end of the sperm duct; their blood contains morula cells. Moreover, in *P. formosana* (= *P. orientalis*), fusion of stolons even of the same colony does not occur in natural growth; but experimental fusion of stolon-pieces is easily attained without reference to their origin, indicating the absence of colony specificity in tissue compatibility (Mukai and Watanabe, 1974). On the other hand, in *P. japonica* and *P. sagamiensis,* stolons of the same colony always fuse in natural growth; but those of different colonies sometimes fuse and sometimes do not, thus indicating the presence of colony specificity (Koyama and Watanabe, 1981, 1982). These differences probably reflect phylogenetic relationships. *P. formosana* seems to be more primitive than the other two species.

In *P. sagamiensis*, the testis is lobulated but forms as an irregular mass. This fact suggests that this species is intermediate between *P. formosana* and *P. japonica* (*cf.* Tokioka, 1953). However, the formation of the brood pouch and the mode of brooding clearly show that *P. sagamiensis* is more advanced than *P. japonica*. It may be concluded that the faculty of terminal budding in *P. japonica* and that of replacement budding in *P. sagamiensis* have been acquired independently by respective species after their separation.

We believe that similar studies of many other species of the Perophoridae will be very helpful in elucidating the phylogenetic relationships within the Perophoridae and those between this and related families.

ACKNOWLEDGMENTS

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AGING IN MODULAR ORGANISMS: ECOLOGY OF ZOOID SENESCENCE IN *STEGINOPORELLA* SP. (BRYOZOA: CHEILOSTOMATA)

STEPHEN R. PALUMBI[1] AND J. B. C. JACKSON[2]

[1]*Department of Zoology, University of Washington, Seattle, WA 98195,* [2]*Department of Earth and Planetary Sciences, The Johns Hopkins University, Baltimore, MD 21218, and*
[1,2]*Discovery Bay Marine Laboratory, Discovery Bay, Jamaica W.I.*

ABSTRACT

The modular construction of clonal plants and animals can lead to physiological deterioration of some clonal components independent of the age or size of the clone as a whole. As a result, senescence does not invariably lead to death (as is the case for solitary organisms), but rather to a patchwork of colony areas exhibiting differing degrees of modular deterioration. In Jamaican cryptic reef environments, older zooids of the cheilostome bryozoan *Steginoporella* sp. exhibit deterioration in feeding and regenerative abilities, are more heavily fouled by epibionts, and contain more stored metabolic waste products when compared to younger zooids. In particular, slower regeneration of broken colony margins in proximal sections allows other encrusting species, which are normally overgrown by the vigorous distal sections of *Steginoporella* sp., to overgrow senescent sections. Thus, although *Steginoporella* sp. is the most abundant bryozoan in Jamaican cryptic reef environments, it can occupy space only ephemerally. Evidence suggests that in *Steginoporella* sp. and ecologically similar species, the disadvantages of proximal senescence are offset by increased translocation of energy or nutrients to vigorously growing distal colony sections.

INTRODUCTION

Hamilton (1966) has shown that pleiotropic genes with deleterious effects later in life could accumulate even in populations of nominally immortal species, leading to an age-dependent increase in the probability of death (*i.e.*, senescence, Medawar, 1957). Although Hamilton considered only solitary organisms, he demonstrated that even in species with indeterminate growth whose fecundity increases exponentially with age, senescence will "tend to creep in" (Hamilton, 1966, p. 25). Many clonal organisms (*e.g.*, hermatypic corals, bryozoa, many grasses) are characterized by indeterminate growth (Harper, 1977; Jackson, 1977) and size-dependent fecundity (Hayward, 1973; Hayward and Ryland, 1975, Yoshioka, 1982). As a result, senescence theory should apply to clonal as well as solitary organisms (Palumbi and Jackson, in prep.).

The modular construction (*sensu* Chapman, 1981) of clonal organisms allows senescence to be studied on two levels: the clone as a whole, and the modules making up the clone. Hamilton (1966) and Williams (1957) have suggested that organisms with low adult mortality rates and increasing adult fecundity will have low senescence rates, and greater longevity. The steeply declining age-specific mortality curves displayed by virtually all clonal organisms studied to date (*e.g.*, Hughes and Jackson,

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1980; Bak *et al.*, 1981; Porter *et al.*, 1981) suggest that senescence rates for clonal organisms should be dramatically lower than senescence rates of most solitary organisms. As a result, clones may be functionally immortal when examined over conventional physiological or ecological time scales (days to years). Senescence may only occur in what is normally considered an evolutionary time frame (millenia).

Clonal organisms, however, frequently exhibit senescence of component modules. Proximal, older modules (*sensu* Chapman 1981) deteriorate and die concomitantly with continued growth and vigor of younger, more distal modules. Such asynchronous senescence within the same genome runs counter to predictions made by Williams (1957) that senescence should be synchronous everywhere within an organism. This is because the modules of clonal organisms show some degree of physiological independence from one another (Harper, 1977; Chapman, 1981). Thus, senescent decline in one module need not necessarily affect the physiology of any other.

While studying the biology and population ecology of cryptic reef communities, we observed physical and functional deterioration (*i.e.*, senescence) of older zooids of *Steginoporella* sp., an encrusting cheilostome bryozoan. Although partial and complete senescence of clonal organisms has been noted (*e.g.* Watt, 1947a, b; Ashby and Wangerman, 1951; Crowell, 1953), little detailed information is available on senescence of modular components of clonal organisms.

Here we document some of the physiological changes associated with senescence of proximal zooids in *Steginoporella* sp. These changes fall into two broad classes (Medawar, 1957), those intrinsic to zooids or colony sections (*e.g.*, accumulation of metabolic wastes [brown bodies], regeneration rate and polarity, feeding rate) and those which result from increased susceptibility to agents external to the clone (*e.g.*, surface fouling, zooid damage). We present methods, results and a brief discussion for each of these types of physiological change, and conclude with a general discussion which provides a rationale for why natural selection may favor modular senescence in *Steginoporella* sp. and other ecologically similar species. We also evaluate the effect of modular senescence of *Steginoporella* sp. on competitive and successional patterns within the Jamaican cryptic reef environment.

Materials and Methods

The undersurfaces of foliaceous corals such as *Montastrea* and *Agaricia* spp. provide insular substrata for a wide variety of encrusting taxa (Jackson and Winston, 1981, 1982). Sponges, Bryozoa and algae occupy most of the available space (Jackson and Winston, 1982; Palumbi unpubl. data). Competition occurs by overgrowth and is characterized both by competitive networks (Jackson and Buss, 1975; Buss and Jackson, 1979) and reversals (Jackson, 1979). In fact, no single species in this system wins in all of its competitive encounters (Jackson, 1979).

All experiments were carried out from June through September 1979 at the Discovery Bay Marine Laboratory in Jamaica. Field experiments were conducted at West Rio Bueno Harbour using SCUBA at depths of −10 to −20 m. *Steginoporella* sp. (hereafter referred to by its generic name), the most abundant bryozoan under plating corals at our study sites (Jackson, in prep.), is an undescribed species previously confused with *S. magnilabris* (Jackson and Buss, 1975; Jackson and Palumbi, 1979). It is identical to *Steginoporella* sp. nov. described in Buss and Jackson (1979) and Jackson and Winston (1981, 1982). Descriptions of the study site are available in Jackson (1979), Buss and Jackson (1979), and Jackson and Winston (1982).

Brown body accumulation

Brown bodies are formed in Bryozoa when a polypide regresses after non-excretable wastes accumulate in the polypide gut wall (Gordon, 1977). The brown body itself is the packaged wastes of the degenerated polypide, and either remains in the coelom of the zooid when the new polypide is formed, or is included in the digestive tract of the nascent polypide and is eventually defecated (Gordon, 1977). If retained, brown bodies accumulate with every degeneration-regeneration cycle of the polypide. They are easily counted, and are a convenient measure of the relative physiological age of *Steginoporella* zooids.

Tissue plugs were removed from *Steginoporella* colonies with a 4 mm diameter cork borer. The number of intact zooids was counted (range, 10–15), and the condition of the colony surface was determined by microscopic examination. Tissue plugs from *Steginoporella* colonies were then subjectively grouped into "fouled" and "unfouled" categories on the basis of the degree of epibenthic fouling of colony surfaces. Fouled areas were brown and were overgrown by diatoms, filamentous algae, or polychaete worm tubes. Unfouled areas, by contrast, were bright red and showed no fouling of the colony surfaces. Any tissue plug which could not be unambiguously placed in either category (<5% of total) was not used in subsequent analyses which depended on such categorization. Following decalcification in 10% acetic acid for 2–4 hours, the plugs were dissected and the number of brown bodies counted. Brown bodies in *Steginoporella* were unaffected by this treatment. Approximately 200 plugs were examined in this way from 35 colonies chosen at random. For five additional colonies, five tissue samples were taken at regular intervals along a haphazardly placed proximal-distal transect.

Feeding

Feeding was observed using a movable dissecting microscope mounted over a 200 l sea water aquarium. Specific colony areas (*ca.* 28 mm^2) at varying distances from the colony edges were examined on 5 successive days for the percent zooids actively feeding at the time of observation. After the final census, a 4 mm diameter sample was removed from the center of each area for brown body determination. Colonies were acclimated in the aquarium for 30 minutes initially, and for an additional 10 minutes between observations. The order of site examination was randomized over successive days to eliminate bias due to colony disturbance during the course of each feeding session.

Lesion distribution and regeneration

Steginoporella colonies grow as fan-like encrustations spreading directionally away from their point of settlement. Twenty-one colonies of *Steginoporella* ranging in size from *ca.* 200 to 9000 zooids were collected and examined microscopically for signs of colony damage. Only lesions involving skeletal injury were recorded. The estimated number of damaged or missing zooids was used as a measure of lesion size. Each colony was mapped, and shattered colony margins (those colony edges bordered by broken zooids) were measured as a percentage of the total observable (*i.e.*, not overgrown) colony margin. In the colonies examined, colony areas were designated as "fouled" or "unfouled" as above. Colony damage (both intracolonial lesions and broken margins) was partitioned into that which occurred in fouled versus unfouled sections.

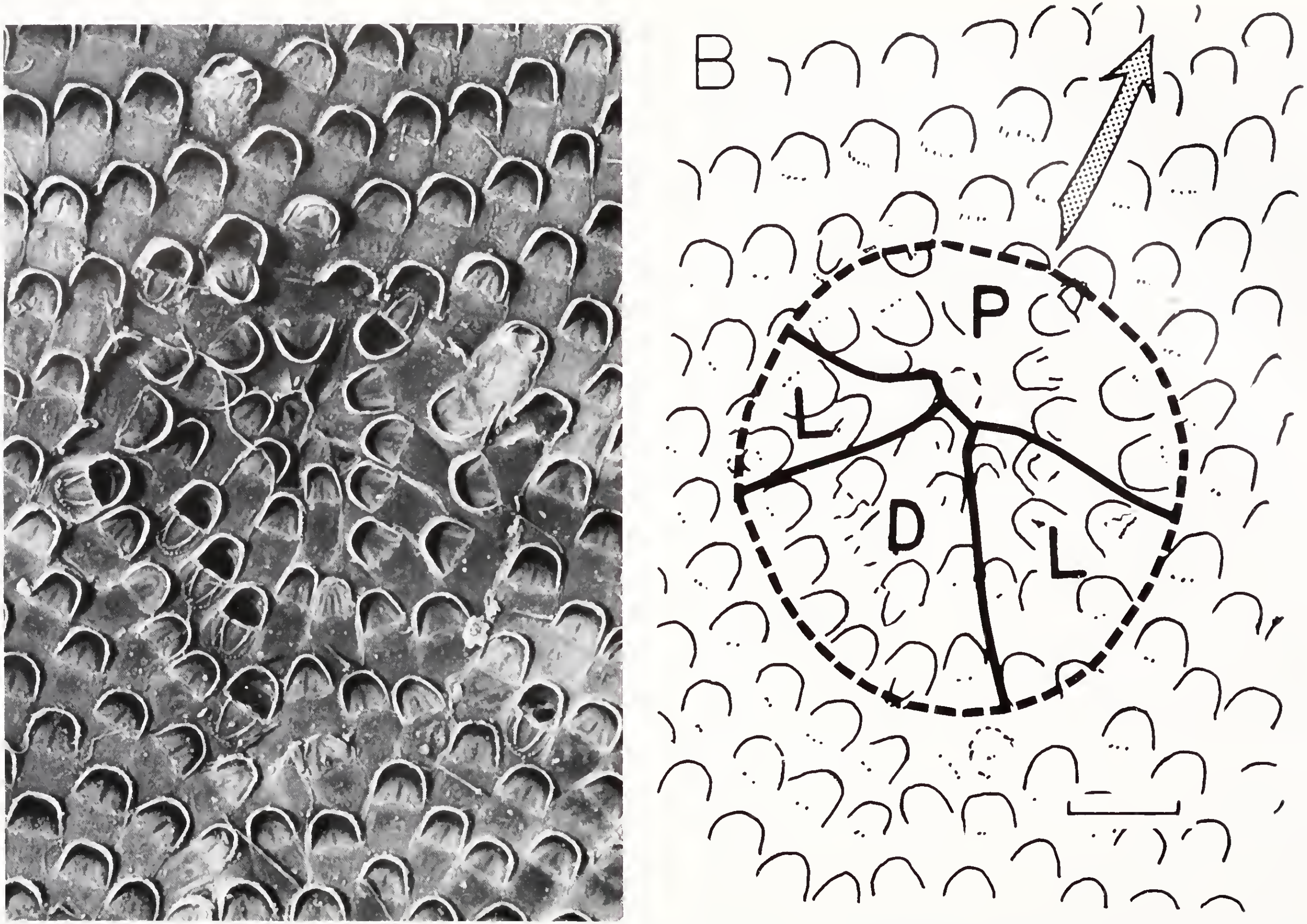

FIGURE 1. A. Photograph of a regenerating lesion in a distal section of a *Steginoporella* sp. colony 27 days after lesion placement. B. Schematic diagram of Figure 1A, dividing regenerated sections into those representing distal (D), proximal (P) and lateral (L) regeneration of the lesion. See text for criteria for these designations. The border of this experimentally placed lesion is shown by a dotted line. The arrow shown designates the proximal-distal axis of the colony. The scale bar represents 1 mm.

Regeneration experiments were initiated by placing one hundred 4 mm diameter, circular lesions in 28 *Steginoporella* colonies. Fifty lesions were placed in distal colony sections; 50 in proximal sections. The tissue plugs removed during lesion placement were saved and analyzed for the number of zooids, the number of brown bodies, and plug surface condition (fouled vs. unfouled). Regeneration was monitored photographically every three days for 7 weeks. Regenerated areas were quantified by projecting slides onto a paper screen, tracing regenerated areas and the boundaries of the original lesions, and weighing the cutout tracings.

Regeneration into circular lesions was further subdivided into 3 groups based on budding direction of regenerating sections. Buds may arise from *i*) the proximal side of the lesion and grow distally (distal regeneration), *ii*) from the distal side of the lesion and grow proximally (proximal regeneration), or *iii*) from the sides of the lesion and grow laterally (lateral regeneration) (Fig. 1). The relative contribution of these three types of regeneration was established for each lesion by subdividing the total area regenerated into the appropriate categories.

RESULTS

Brown bodies

Most *Steginoporella* zooids contain one or more brown bodies; some zooids contain more than twenty. Figure 2 shows the numbers of brown bodies per zooid in dissected tissue plugs plotted against distance of those plugs from the distal edge

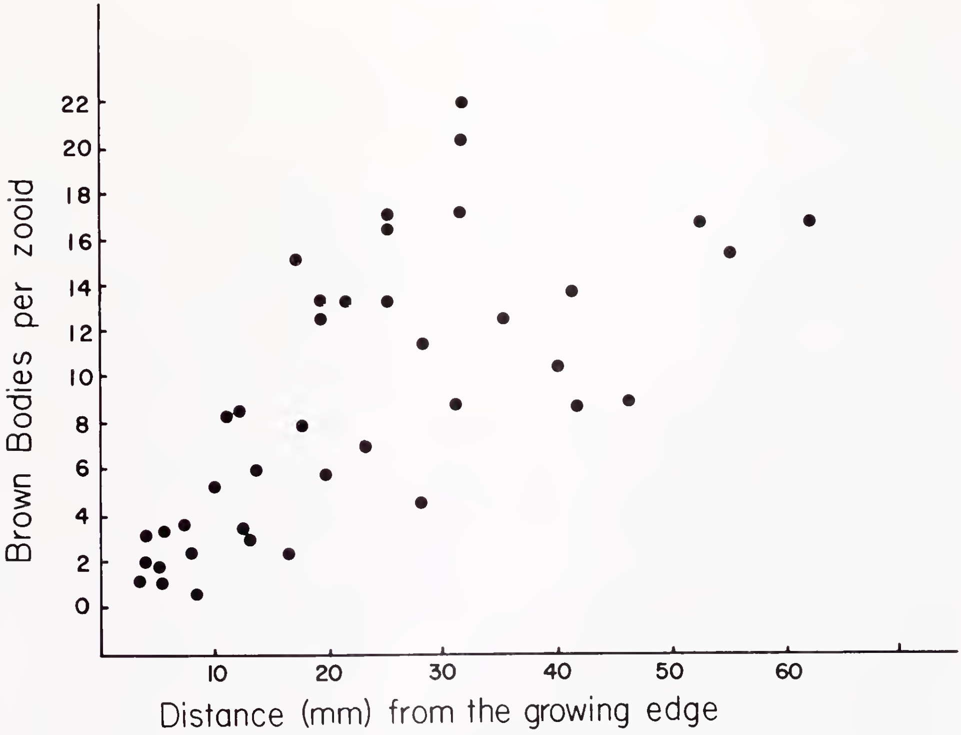

FIGURE 2. The number of brown bodies per zooid in colony sections at varying distances from the colony growing edge (Spearman rank correlation coefficient, $P < 0.001$).

of the colony. Brown bodies significantly increase in frequency in proximal colony areas (Spearman rank correlation; $P < .001$).

Analysis of brown body accumulation in tissues sampled from diverse colony areas showed that areas designated as "fouled" exhibited more brown bodies per zooid (median = 6.3 brown bodies per zooid, n of plugs = 85, n of zooids = 1105) than did areas subjectively labelled "unfouled" (median = 1.5, n of plugs = 75, n of zooids = 975, distributions distinctly non-gaussian; medians different, $P < 0.001$, Wilcoxon 2-sample test). Large numbers of brown bodies may crowd the polypide, hindering its function, or preventing further rejuvenatory cycles. Thus, the operational lifespan of a zooid may in some cases be restricted by the number of brown bodies that it can hold.

All colonies in which tissue plugs were sampled along transects exhibited monotonic increases in brown body accumulation in sequentially more proximal samples. The substantial scatter evident in Figure 2 is a result of marked intercolony variation in brown body accumulation. The degeneration-regeneration cycle which produces brown bodies may be influenced by adverse environmental conditions (Gordon, 1970, 1977; Cummings, 1975), food supply (Ryland, 1976), or the buildup of toxic waste products in the polypide (see Gordon; 1977). Environmental differences, such as temperature, turbulence, or sedimentation can affect the physiology of whole colonies (Menon, 1972) or parts of colonies (Boardman *et al.*, 1969; Boardman and Cheetham, 1973), and the variability in brown body accumulation may reflect differences in the microenvironments to which *Steginoporella* colonies are subjected. Unfortunately, few data are currently available on the temporal or spatial variation in the physical environment under coral plates.

Feeding

Feeding activity is negatively correlated with both brown body accumulation (Spearman rank correlation; n = 14, $r_s = -.64$, $P < 0.02$) and the distance of the areas observed to the colony growing edges ($r_s = -.73$, $P < 0.01$). Figure 3 shows feeding activity plotted against brown body accumulation. Nearly all zooids in distal portions of colonies (from 2 to 10 mm from the growing edge) actively fed during all observations, whereas as few as 40% of the zooids in more proximal colony areas (15 to 40 mm from the growing edge) were actively feeding.

Interzooidal transfer of nutrients (Bobin, 1964, 1977), and products of polypide regression (Cummings, 1975), plus the high incidence of non-feeding heterozooids (Boardman and Chetham, 1973; Schopf, 1973) suggest that colony wide availability of energy is important in the maintenance and growth of bryozoan zooids. In addition, the feeding behavior of individual zooids is often coordinated, presumably to increase the food intake of the colony as a whole (Banta *et al.*, 1974; Winston, 1977, 1978). Control of zooid feeding, then, does not originate solely within single zooids. Rather, feeding is apparently regulated by the colony as a whole.

Reduction of feeding activity in older zooids may make coordination of feeding currents more difficult, and may serve to reduce the average energy intake of proximal zooids. Whereas in some bryozoa (including some *Steginoporella* species) developing embryos are often housed in non-feeding zooids (Cook, 1964; Silen, 1977; Strom, 1977), proximal zooids in *Steginoporella* sp. do not contain a disproportionate fraction of brooded embryos (Winston and Palumbi, unpubl. data).

Feeding induced water currents may play a role in competition for food (Buss, 1980), and have been implicated in inhibition of epibenthic settlement (Palumbi and Jackson, 1982). Thus, current reduction may decrease the competitive ability

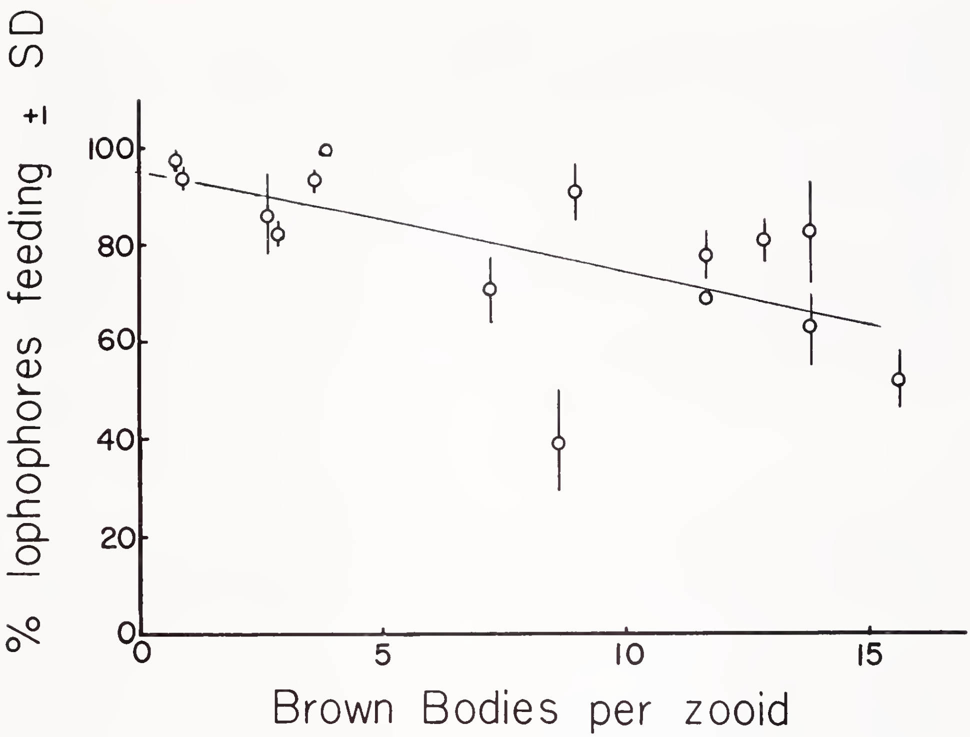

FIGURE 3. The percentages of zooids actively feeding in sections of *Steginoporella* colonies plotted against the average number of brown bodies per zooid in those colony sections. Each point represents an average of daily observations over five consecutive days and an average of the brown bodies in 10 to 15 zooids (Spearman rank correlation, $P < 0.05$).

of older colony areas. In fact, older areas of *Steginoporella* are more commonly overgrown by other organisms than are younger, distal colony sections. Thus, older sections of *Steginoporella* probably contribute less to the colony as a whole, and are more likely to be lost to encroachment by competitors or settlement by fouling organisms than are younger colony sections.

Lesion distribution and regeneration

Ninety-five lesions were observed in 21 *Steginoporella* colonies. Colony areas visually characterized as "fouled" exhibit, on average, a higher percentage of damaged zooids (5.9%) than do areas characterized as "unfouled" (3.6%; $P < .001$ test of percentages, Sokal and Rohlf [1969]) (Table I). Margins bordering older colony areas were nearly always (97%) shattered whereas only 24% of the margins of younger colony sections were broken ($P < 0.001$; Wilcoxon 2-sample test) (Table I).

Experimentally placed lesions in *Steginoporella* can be grouped into two categories: those which began regeneration almost immediately (within 3–6 days), and those which only slowly regenerated (if at all). Lesions in this latter class were found to be those visually characterized as "fouled", and were always in proximal colony areas. Furthermore, 95% had more than 3 brown bodies per zooid. By contrast, 90% of the lesions which quickly regenerated were in distal, unfouled colony sections and had fewer than 3 brown bodies per zooid. Regeneration was much faster in

TABLE I

Characteristics of proximal (older) and distal (younger) areas of Steginoporella *sp. colonies*

	Distal	Proximal	Tests and significance
Physical characteristics:			
color	salmon red	dark brown	
fouling	slight	heavy	see Jackson, 1979
% damaged zooids	3.6	5.9	test of percentages; $P < 0.001$
% shattered margins	24	97	Wilcoxon 2-sample test; $P < 0.001$
median # brown bodies/zooid	1.5	6.3	Wilcoxon 2-sample test; $P < 0.001$
Functional characteristics			
average % lophophores feeding	90	75	Spearman rank correlation coefficient: n = 14; feeding vs. brown bodies, $P < 0.02$; feeding vs. distance to growing edge, $P < 0.01$.
% regeneration after 36 days (±SEM)	80 ± 7	16 ± 6	Wilcoxon 2-sample test; $P < 0.001$
regeneration rate (mm^2/day)	.274 ± .03	.060 ± .02	Spearman rank correlation coefficient: n = 31; regen. vs. brown bodies, $P < 0.01$; regen. vs. distance to growing edge; $P < 0.01$.
% holes completely regenerated after 36 days	60	9	test of percentages; $P < 0.001$
% holes showing no regeneration after 36 days	5	72	test of percentages; $P < 0.001$
% regeneration in			
distal	48	24	
proximal directions	18	47	2-way ANOVA; $P < 0.005$
lateral	35	29	
wins/losses in competitive overgrowths	6.8	0.1	2 × 2 contingency table; $P < 0.001$ (Jackson, 1979)

areas with lower (<3) rather than higher (>3) brown bodies per zooid (Table I, Palumbi and Jackson, 1982), and the regeneration rate was negatively correlated with both the number of brown bodies per zooid in that colony area (Spearman rank correlation, n of lesions = 31, $r_s = -.67$, $P < .01$), and with distance from the colony margin ($r_s = -.56$, $P < .01$).

Feeding reduction alone can not adequately account for the low regenerative capacity of proximal zooids. Percentages of lophophores feeding in proximal colony areas are 10%–60% lower than in distal colony areas, whereas regeneration rates in the same areas differ more than four-fold (Table I).

Natural patterns of lesion distribution parallel these experimental results. Distal areas of *Steginoporella* colonies exhibit fewer lesions and less extensive shattering of colony margins than do proximal areas. This is probably a dual reflection of lowered opportunity for colony damage in the younger colony sections, and slower lesion regeneration in older sections.

Regeneration polarity in areas with lower versus higher numbers of brown bodies per zooid is shown in Table I. These regenerative budding patterns suggest that energy allocation to growth and repair is a complex function of energy flow patterns within *Steginoporella* colonies. Adjacent zooids in a bryozoan colony are intimately connected through pores, and nutrients can be shunted (in as yet unknown quantities and rates) from one portion of a colony to another (Bobin, 1964, 1977; Ryland 1979). Almost all lesions (95%) in distal colony areas commenced regeneration, and did so in a predominantly distal direction (Table I). That is, regenerated zooids were first budded on the proximal side of the lesion, and regeneration was primarily a result of growth in the distal direction (see Fig. 1). This probably reflects a prevailing proximal-to-distal orientation of energy flow (translocation polarity) within colonies. By contrast, lesions in proximal colony regions regenerated infrequently (72% failed completely). When regeneration did occur, budding was primarily in the proximal direction, suggesting that regeneration requires shunting of energy from more distal colony sections. Thus, the high failure rate of proximal lesions may in part reflect a low potential for reversal of normal translocation polarity.

Discussion

Proximal senescence in clonal organisms

The consistent differences between young and old zooids summarized in Table I suggest that deterioration of proximal portions of *Steginoporella* colonies is irreversible, and represents zooid senescence. A *Steginoporella* colony is a patchwork of areas; the older sections are, on the basis of the regeneration data, more vulnerable, and on the basis of the feeding data, less productive than are younger sections. Survival of any colony section is ultimately limited by senescence, at least partially because of decreased ability to defend itself against encroachment by other organisms. Crowding within zooids containing many brown bodies may also play a role in the loss of zooid function.

Thus, *Steginoporella* survives through a balance of spatially partitioned growth and decay. High overgrowth ability of distal margins promotes increase in colony size whereas deterioration of more proximal zooids tends to decrease colony size. In this way, a zygotic individual (clone) may survive for many years, but steadily "moves" across the substratum (Jackson and Winston, 1981).

This growth pattern has important consequences in the Jamaican cryptic reef environment. Proximal senescence of *Steginoporella* zooids and associated loss of competitive ability (Jackson, 1979) may result in a cyclic pattern of space occupation. Many species are overgrown by the vigorous distal margin of *Steginoporella* colonies, but can overgrow senescent colony sections (Jackson, 1979). In fact, proximal senescence in *Steginoporella* accounts for 9 of the 26 instances of competitive indeterminacy recorded in this community (Buss and Jackson, 1979). Thus, the growth of *Steginoporella* over a particular area eventually results in local opportunity for recruitment or encroachment by practically any other resident of the cryptofauna. Successional development (*e.g.*, Dean, 1981; Field, 1982) is slowed or circumvented since *Steginoporella* occupies space only ephemerally.

Anecdotal observations suggest that this pattern of partial senescence is phylogenetically widespread (although by no means universal) among clonal organisms including corals (Hildemann 1978), hydroids (Campbell, 1968), bryozoans (Bronstein, 1939; Ryland, 1979), ascidians (Sabbadin, 1979; Birkeland *et al.*, 1981), fungi (Carlile, 1979), encrusting red algae (Paine *et al.*, 1979), and many other plants (Watt, 1947a, b; Ashby and Wangerman, 1951; Bell, 1974). The possible adaptive significance of partial senescence remains undemonstrated, and may vary between taxa. In *Steginoporella,* the marked translocation polarity indicated by the regeneration experiments coupled with high distal growth rates (Winston and Jackson, in prep.) suggest that proximal zooid senescence and subsequent distal translocation of released nutrients enhances the growth rate and overgrowth ability of this species.

The growth pattern of *Steginoporella* is closely paralleled by growth in some filamentous fungi (Division Ascomycetes). These organisms are clonal, indeterminate growers with hyphae (fungal growth processes) periodically compartmentalized by incomplete septa (Webster, 1980). Cytoplasm, nuclei and sometime whole mitochondria can pass between compartments, and translocation (in at least some groups) is primarily proximal-to-distal (Fencl, 1978). Extensive laboratory work has resulted in detailed documentation of proximal senescence in these taxa (Holliday, 1969; Fencl, 1978). Genetic analyses indicate that some senescent symptoms result from cytoplasmic effects inherited maternally, including several mitochondrial mutants which quickly spread and debilitate affected colonies (Turner, 1978).

It is premature to extend these results and to speculate on the physiological control of senescence in Bryozoa. Senescence in fungi is frequently associated with the death of the entire colony (Holliday, 1969), whereas in *Steginoporella,* distal colony sections may remain vigorous for years despite proximal senescence (Table 1; Jackson and Winston, 1981). Interzooidal pores in Bryozoa are generally too small to allow organelle migration (Bobin, 1964). Furthermore, the ability of proximal colony sections to occasionally bud functional zooids suggests a more complicated developmental control of senescence in *Steginoporella.* Nevertheless, the parallels between these two divergent taxa are striking.

Staghorn coral, *Acropora cervicornis* (Porter, 1974; Tunnicliffe, 1981) and bracken fern, *Pteridium aquilinum* (Watt, 1947a, b) exhibit similar combinations of apparent senescence (*i.e.*, higher mortality) of proximal modules, very rapid distal growth, and exceptional competitive ability. Like *Steginoporella,* they are commonly the most abundant organisms in their respective habitats (Watt, 1947a; Goreau, 1959) and individual clones may be very long lived (Oinonen, 1967; Jackson and Winston, 1981; Tunnicliffe, 1981). In these organisms, proximal senescence may free colony resources for increased distal colony growth, thus facilitating overgrowths in interspecific encounters and/or allowing increased sampling by these sessile organisms of a heterogeneous or temporally unstable resource (Buss, 1979; Lovett Doust, 1981).

Although much is known of the physiology of proximal senescence in some taxa (especially fungi; Fencl, 1978) little is known of the factors which select for senescent traits. Laboratory descriptions of the process of senescence can not by themselves reveal the underlying causes of aging. An understanding of the ecological implications of proximal senescence in a broad range of species may, when coupled with laboratory investigations, provide a clue to the nature and control of senescent decline.

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SPECIFIC IDENTIFICATION AND ASSESSMENT OF DISTRIBUTION AND ABUNDANCE OF EARLY PENAEID SHRIMP LARVAE IN THE GULF OF CARPENTARIA, AUSTRALIA

PETER C. ROTHLISBERG, CHRISTOPHER J. JACKSON, AND ROBERT C. PENDREY

Division of Fisheries Research, CSIRO Marine Laboratories, P. O. Box 120,
Cleveland, Queensland 4163, Australia

ABSTRACT

Methods for obtaining eggs and rearing larvae of penaeid shrimp at sea, in remote areas, were developed and used to build a comprehensive larval reference collection for taxonomic purposes. Because of the large amount of morphological variation within species and character overlap between species, a multivariate numerical identification technique, discriminant analysis, was tested using larvae of the four species of *Penaeus: p. esculentus; P. latisulcatus; P. merguiensis;* and *P. semisulcatus,* in our reference collection. The overall accuracy of the technique is high (>85%) and can be increased by narrowing the range of natural morphological variation considered, at the expense of decreasing the number of larvae positively identified. Application of the technique to the first zoeal larvae in our plankton collections from the Gulf of Carpentaria, Australia, shows discrete, discontinuous larval distributions which delimit the spawning activity of the four species to a degree not possible by sampling and histological examination of the adult shrimp.

INTRODUCTION

The family Penaeidae makes up approximately 70% of the world's prawn catch (estimated from Table II in Wickins, 1976). These penaeid stocks are also characterized by very large fluctuations in size (Kirkegaard, 1975), with little apparent relationship between spawner abundance and recruitment strength (Neal, 1975; Rothschild and Gulland, 1982). This is not surprising considering that most commercially important species are fecund, short-lived and have a complex life cycle which involves a variety of distinct habitats (Kutkuhn, 1966; Garcia and Le Reste, 1981). After the demersal eggs hatch the pelagic larvae go through a series of larval stages which are lecithotrophic, herbivorous and carnivorous before reaching their nearshore or estuarine nursery areas and settling out of the water column. Because of the high fecundity and complex larval life history, it is probable that a large amount of the year-to-year variability in stock size is accounted for by factors affecting larval and postlarval mortality. Detailed studies of the larval ecology, as well as precise assessment of reproductive activity based on distribution and abundance of early larval stages, have been hampered by the inability to identify the early larval stages found in the plankton (Racek, 1959; Temple and Fischer, 1967; Kutkuhn *et al.,* 1969; Subrahmanyam, 1971; Sandifer and Eldridge, 1976).

Methods for identifying the genera of penaeid zoea larvae are well established. The characters which have been found to be most useful relate to the setal distri-

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All measurements are presented in ocular divisions (O.D.) where 1.0 ocular division = 0.118 mm.

bution on the endopod of the second antenna (Cook, 1966a; Hassan, 1974). To date however, there has been no success in identifying specific characters of larval stages. Cook (1966b) reared larvae of *P. aztecus* and *P. duorarum*, but could not detect a difference in either setation or ratios of various morphological features. Subrahmanyam (1971) studied larvae taken from the plankton, and although he could separate two species of *Sicyonia* using setation on the 2nd antenna endopod, he was unable to subdivide three species of *Penaeus* (*P. fluviatilis* [= *P. setiferous*], *P. aztecus*, *P. duorarum*) or two species of *Trachypenaeus* (*T. similis* and *T. constrictus*) on the basis of morphology or morphometry. He suggested that studies at the biochemical or molecular level may be necessary to achieve this. Cook and Murphy (1971) reared *P. aztecus*, *P. duorarum* and *P. setiferus* and found them to be identical in setation and other major morphological characteristics. They also searched unsuccessfully for differences in various morphological ratios. Courties (1976) reared *P. indicus* and *P. semisulcatus* to 1st zoea, and could not distinguish them. Most recently, Motoh (1979) and Motoh and Buri (1979) reared larvae of *P. monodon* and *P. merguiensis* and compared them with *P. japonicus* described by Hudinaga (1942). They listed several differences between the species, chiefly the nature of the supraorbital spine in 2nd zoea, and the segmentation and setation of the 2nd maxilliped in several stages. However, they mentioned that these differences might well be due to individual or local variation, or to the effects of different rearing conditions. In support of this idea, Motoh and Buri (1979) drew attention to several differences in the description of the larvae of *P. merguiensis* raised by them and the same species raised by Raje and Ranade (1972). Many of the characteristics of the early larval stages are subject to variation induced by growth during an instar (Hudinaga, 1942; Motoh, 1979; Motoh and Buri, 1979) as well as the possible effects of environmental variability.

We therefore decided to seek a statistical method for identifying larvae which would take into account the variability in larval characters, and would use a number of characters simultaneously. Although the clustering multivariate techniques of numerical taxonomy have been used to identify unknown animals by pooling the morphological data for both knowns and unknowns and applying a clustering program to this data matrix (*e.g.*, Campbell, 1973; Colwell *et al.*, 1973), this approach is most useful when the aim is to study the nature of the taxonomic boundaries involved. When the problem is solely identification, discriminant analysis is the preferred technique. A full treatment of the theory and application of discriminant analysis is given in Lachenbruch (1975).

In this paper we describe the methods used to obtain reference specimens of penaeid larvae and test the discriminant analysis with the reference material. We then apply the technique to the first zoeal (= protozoeal) larvae from plankton samples obtained on one cruise in the Gulf of Carpentaria, Australia in March, 1977.

MATERIALS AND METHODS

Ovigerous females—collection and spawning

Ovigerous females were sought on commercial shrimp grounds, using chartered trawlers. Ripe and healthy females were sorted from the catch, as quickly as possible, and placed in clean sea water which was obtained by bucketting. Sea water from deck hoses was avoided because of elevated temperatures and the possibility of metallic or other contaminants in the ship's plumbing. After the whole catch had been sorted the females that had been chosen were re-examined. Over the period of sorting, weak and moribund animals became more obvious. Vigorous heartbeat and action of the scaphognathite were the primary criteria for health. The ripeness of the ovaries was assessed subjectively. This assessment was usually a combination of depth of color, granular appearance, and degree of fullness as estimated by the size of the ovarian outpockets on the dorsal side of the first abdominal segment. Females meeting these criteria were placed in the holding tanks (see section under larval rearing). Through the night's trawling, females were monitored at approximately half-hour intervals for health and spawning. Culling was continuous, the ripest females from each trawl replacing less ripe animals. Careful selection of ripe females resulted in a high proportion of spontaneous spawnings; induction methods were not found to be necessary. Normally spawning was extremely rapid and was manifested by a coat of bubbles on the top of the holding container and a thick band of pink proteinaceous foam around the edge of the container at the water line. The presence of eggs was verified by dipping from the container with a clear beaker. The eggs were usually left for about one half hour so that they would be sufficiently robust for subsequent handling, and then siphoned from the holding containers successively through a 500-μm screen which retained most fecal matter and other debris, and a submerged 90-μm screen which retained the eggs. They were washed several times before being pipetted into larval rearing containers or egg transport modules.

Shipboard larval rearing

Because of the limited space on chartered trawlers, a rack capable of holding four tiered 600 mm square PVC baths of running sea water, was constructed of bolted channel section galvanized steel (Fig. 1;B). This portable apparatus could be dismantled and shipped by air to remote areas and moved easily from a vessel to a shorebased rearing facility. The top three water baths, used for larval culture (Fig. 1;1, 2, 3), each contained 36 tall-form 400 ml beakers, containing 250 ml of sea water. The fourth water bath (Fig. 1;4) was used for brine shrimp culture, and held 16 one litre beakers, each containing 700 ml of sea water. A fifth tray, without flowing sea water, held 36 spare 400 ml beakers. All beakers had glass petri dish lids to prevent splash contamination. The water level in each tray was maintained by a weir at a level slightly below the water level within the beakers. Water flow (3 l/min) was directed through the tray and past each beaker, in series, by baffles. This flow rate was sufficient to prevent any noticeable temperature differential across the water bath and the baffles prevented water surge as the boat rolled.

Surface sea water was supplied by a 25 mm centrifugal electric pump (Fig. 1;C). Water flow was controlled by a manifold of 6 valves, four controlling the larval water baths and two regulating the surplus water which was used to cool the female holding tanks (Fig. 1;D). Aeration to the rack was provided by a 4-diaphragm aquarium aerator (Fig. 1;A). The manifold for each tray was supplied by a separate diaphragm, in order to localize the effects of a diaphragm failure. Air supply to individual larval rearing beakers was regulated at 35 ml/min by a 230 mm length of 0.38 mm ID polyethylene tubing attached to the rigid 25 mm diameter PVC air manifold. Each beaker's aerator was weighted with a section of thick walled glass capillary tubing. The airflow to the brine shrimp beakers was regulated at 60 ml/min by a 150 mm length of the 0.38 mm ID tubing.

Larvae were fed a mixture of three species of marine phytoplankton (*Thalas-*

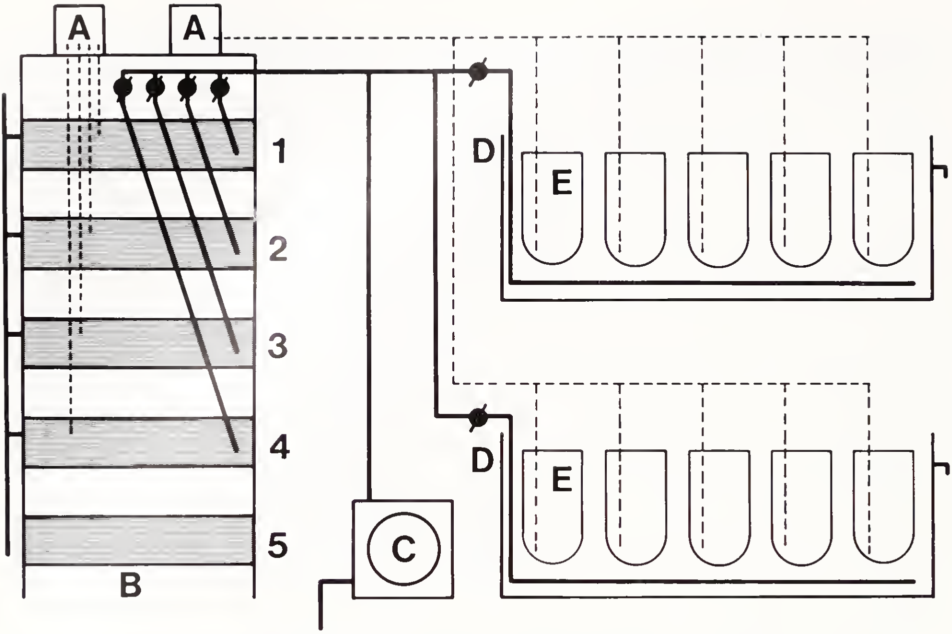


FIGURE 1. Shipboard larval rearing rack (B), with three water baths (1, 2, 3) for larval rearing, one (4) for rearing brine shrimp and storage (5) for spare larval rearing beakers. Female isolation chambers (E) in circulating water baths (D). Pump (C) to supply surface sea water for temperature regulation of both the larval incubation trays and the female holding containers. Two aerators (A) to supply air to individual larval and adult containers. See text for details of construction and operation.

siosira pseudonana, Isochrysis galbana, Tetraselmis (= Platymonas) suecica) that previously had been grown, harvested, concentrated to predetermined cell concentrations, and frozen with dimethyl sulfoxide (DMSO) as a freeze-thaw protectant (Brown, 1972) in 10 ml plastic test tubes (Pendrey, unpublished). The algae were thawed and diluted to pre-determined feeding aliquots, on board, and fed to the larval cultures four times per day. Brine shrimp cultures were maintained on board for feeding to the older larvae (mysis and postlarvae) along with the algal diet.

The sides of the rack were covered by 70% shade cloth to keep the cultures cool and prevent direct sunlight entering the larval containers. The top of the rack had a pitched waterproof canvas roof to protect the aerators from rain and salt spray.

Female-holding and spawning containers

Two large fiberglass tanks (500 × 1200 × 600 mm) (Fig. 1;D) with flow-through sea water circulation were used as water baths for maintaining gravid females at surface sea water temperatures. The females were held in 9 l polyethylene bags (Fig. 1;E) (10 bags per water bath) held open with a polyethylene ring and suspended from a metal rack across the top of the water bath. A separate aquarium aerator was used for these female holding tanks. Airflow to each plastic bag was regulated by a 25 mm length of 0.38 mm ID polyethylene tubing. The standing water in each bag was treated with 0.1 ppm EDTA to suppress bacterial growth. Each bag could hold up to five large females of the same species. The female-holding tanks were covered with waterproof canvas to exclude light, rain and fall-out from the ship's diesel exhaust.

Egg transport modules

Under certain circumstances shipboard rearing was not desirable. A method of transporting eggs from spawning locations to our laboratory was developed for these instances. Self-contained modules (Fig. 2) enabled us to airfreight eggs or early larvae. The modules were immersed in 12–16 l of sea water in an insulated polyethylene container. Air was provided by a battery operated aerator which would run for more than 48 hours on one set of batteries. An air lift forced the water through a 200 g bed of coarse activated charcoal to remove any organic toxins. The water then passed through a 20 mm thickness of Dacron wool to remove detritus and then through a 142-μm nylon mesh which supported the eggs. The water flowing up through the eggs gently agitated and aerated them and prevented dense anaerobic clusters from forming. Any naupliar stages that hatched in transit were retained in the incubation chamber formed by a clear 90 mm diameter acrylic plastic tube and a removable 142-μm mesh cap. The whole unit was immersed so that water could flow unimpeded over the rim of the cap. The pipe to release the air from the airlift was about 40 mm above the water surface. The use of 0.1 ppm EDTA during egg transport in some cases seemed to lead to higher hatching rates.

Larval morphology

Larvae for our reference collection were obtained both from our own shipboard and laboratory rearing, and from other sources (see Results, Reference collection section). Samples of larvae for morphological studies were preserved on several occasions during each larval instar to ensure that the full range of morphological variability for a given instar would be represented.

Larvae were preserved initially in buffered (sodium tetraborate) 10% formaldehyde. They were then transferred via 70% ethanol to a PVA/phenol/lactic acid medium with chlorazol black E (Perkins, 1956) at least two days before any morphological measurements were made. Individual larvae were placed in a drop of PVA medium, on a well slide, without a cover slip. The viscosity of the PVA medium allowed the larva to be arranged easily for subsequent viewing and measurement. Measurements were made using a compound microscope, with 10× ocular equipped with micrometer, and a 10× phase contrast objective. All measurements were recorded to the nearest 0.1 ocular division (1.0 ocular division = 0.118 mm) and all data are presented in ocular divisions (O.D.). Morphological measurements were recorded for a total of 846 1st zoea of four *Penaeus* species (133 *P. merguiensis,* 192 *P. esculentus,* 355 *P. semisulcatus,* and 166 *P. latisulcatus*) from the reference collection. The 14 measurements made (Fig. 3) were: 1) Total length; 2) Carapace length; 3) 1st Antenna length; 4, 5, 6) Lengths of segments of 1st antenna; 7) Diameter of 2nd antenna basis; 8) Length of 2nd antenna endopod; 9) Length of 2nd antenna exopod; 10, 11, 12) Lengths of segments of 2nd antenna endopod; 13) Diameter of abdomen; 14) Diameter of 1st antenna.

Discriminant analysis was performed on the CYBER 76 computer operated by CSIRO Division of Computing Research, Canberra, Australia; the DISCRIMINANT subprogram of the Statistical Package for the Social Sciences (SPSS) software library (Nie *et al.,* 1975) was used. We developed interactive programs for data collection and identification of unknown larvae. These programs were run on a PDP-11 minicomputer.

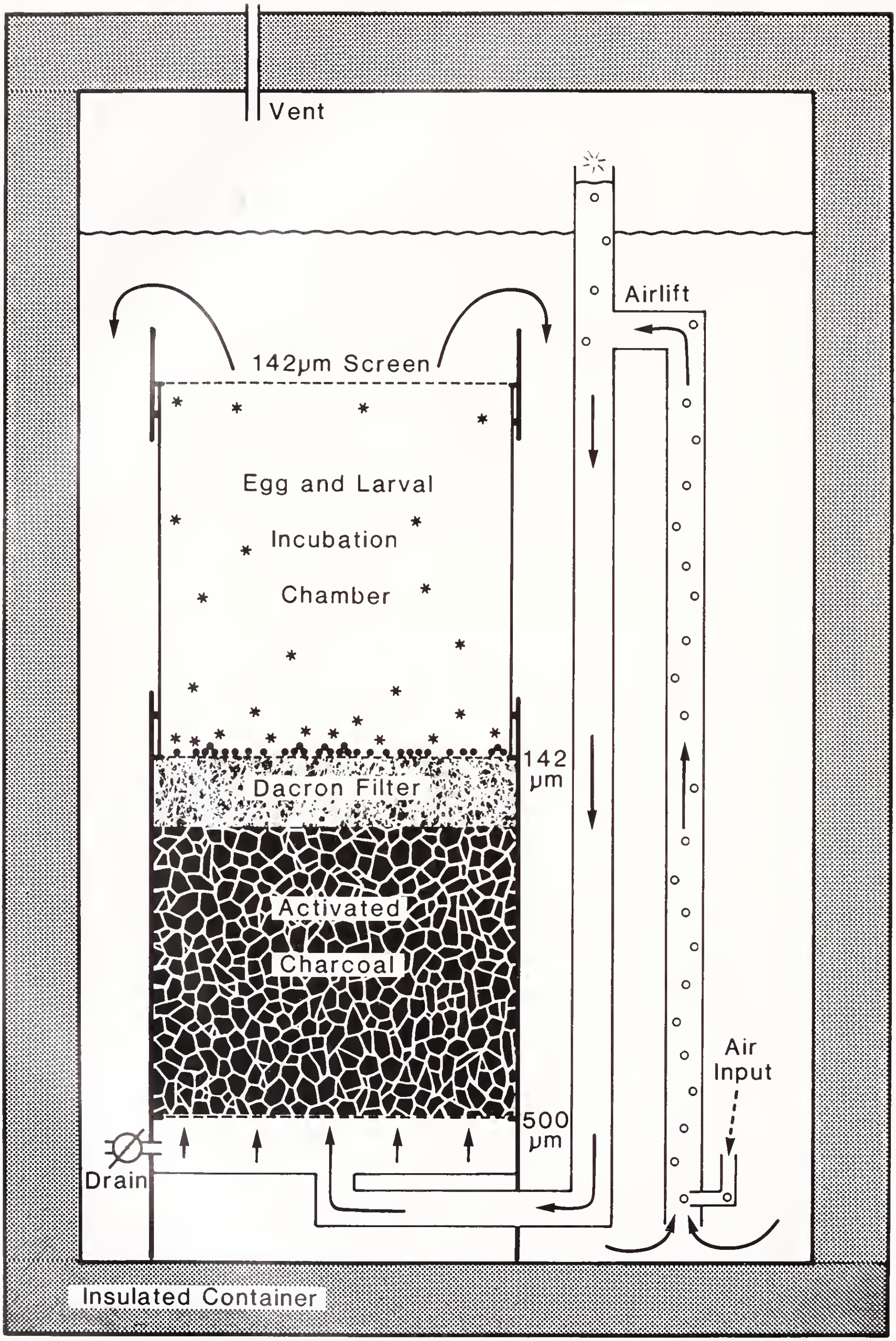
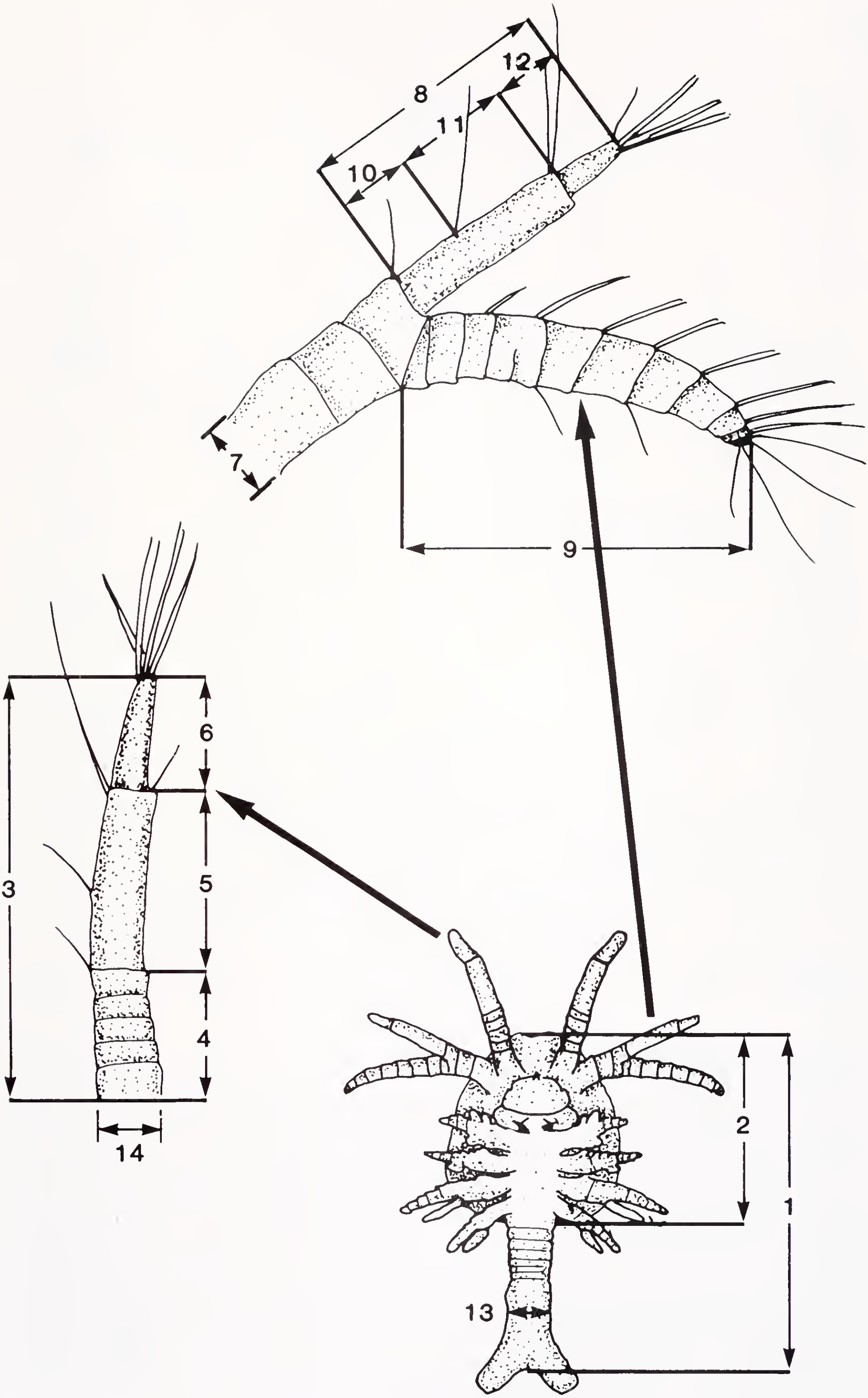

Vent
Airlift
142µm Screen
Egg and Larval
Incubation
Chamber
142
µm
Dacron Filter
Activated
Charcoal
500
µm
Air
Input
Drain
Insulated Container

FIGURE 2. Egg and larval transport modules in an insulated water container. Figure not drawn to scale. See text for details of construction and operation.



8
12
11
10
7
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5
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4
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2
1
13

FIGURE 3. Fourteen morphological characters of the first zoea penaeid larva used in discriminant analysis.

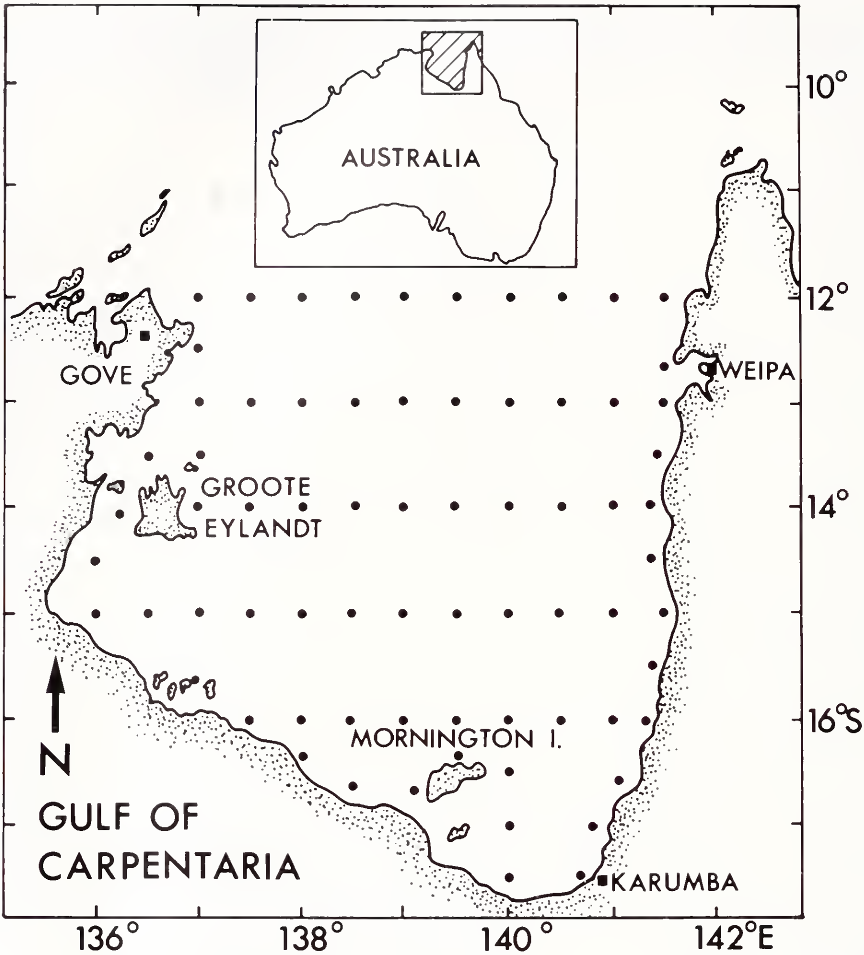

FIGURE 4. Gulf of Carpentaria, Australia. Dots indicate location of plankton samples taken on cruise in March, 1977.

Plankton sampling

Plankton samples were obtained in a manner described by Rothlisberg and Jackson (1982). Paired samples (142 and 500 μm nets), analyzed in this study, were taken between 16 and 30 March 1977 at 70 stations (Fig. 4).

All samples were initially split in half using a Folsom plankton splitter (McEwen *et al.*, 1954). One half of the formalin-fixed sample was subjected to a destructive biomass analysis (Rothlisberg and Jackson, 1982) while the other half was split further, transferred to 2% 2-phenoxyethanol and stored for microscopic analysis. Larvae from the 142 μm samples were sorted from these fractions using binocular dissecting microscopes. Larval numbers were standardized by calculating the volume of water filtered, from calibrated flowmeter readings, and then multiplied by the sample depth, from a time-depth recorder trace, to give larval abundance values integrated under 1 m^2 of sea surface (Kramer *et al.*, 1972).

TABLE I

Species and stage of development of penaeid larvae in reference collection

	Larval stage of development							
		Zoea			Mysis			
Species	Nauplius	1	2	3	1	2	3	Postlarva
Penaeus								
P. esculentus	*	*	*	*	*	*	*	*
P. indicus	*	*	*	*	*	*	*	*
P. latisulcatus	*	*	*	*	*	*	*	*
P. merguiensis	*	*	*	*	*	*	*	*
P. semisulcatus	*	*	*	*	*	*	*	*
Metapenaeus								
M. bennettae	*	*	*					
M. eboracensis	*	*	*	*				
M. endeavouri	*	*	*	*	*	*	*	*
M. ensis	*	*	*	*	*	*	*	*
M. insolitus	*	*	*	*	*			
Trachypenaeus								
T. anchoralis	*	*	*	*	*	*	*	*
T. fulvus	*	*	*	*	*	*		
Atypopenaeus								
A. formosus	*	*	*	*	*			
Parapenaeopsis								
P. cornuta	*	*	*	*				
Metapenaeopsis								
M. novaeguinea	*	*	*	*	*			
M. palmensis	*	*	*	*	*	*	*	*
Genera	6	6	6	6	5	4	4	4
Species	16	16	16	15	13	10	9	9

RESULTS

Larval reference collection

Table I contains the species and stage of larval development we have obtained for our reference collection. All but a few of the species were obtained in the Gulf of Carpentaria using the shipboard rearing facilities. *Penaeus indicus* was spawned in the Gulf of Papua, off Yule Island, and the eggs were shipped to our laboratory in Cleveland, southern Queensland. We have also spawned *P. merguiensis, P. esculentus,* and *Metapenaeus bennettae* on chartered trawlers in Moreton Bay, adjacent to our laboratory. These *P. merguiensis* and *P. esculentus* were used to compare with and supplement the number of larvae we had obtained by shipboard spawning and rearing in the Gulf of Carpentaria. Specimens of *P. merguiensis* have also been obtained from France-Aquaculture, New Caledonia.

In order to enlarge our collection of *P. semisulcatus* larvae we obtained ovigerous females off Cairns, northern Queensland. Unlike the Gulf of Carpentaria cruises during which *P. semisulcatus* was spawned routinely, we had repeated difficulty obtaining viable eggs on board. The ripe ovaries frequently would change color,

TABLE II

Mean, standard deviation and range of 14 morphological characters for the first zoeal instar of four species of Penaeus

Character	*Penaeus esculentus* (n = 192)	*Penaeus latisulcatus* (n = 166)	*Penaeus merguiensis* (n = 133)	*Penaeus semisulcatus* (n = 355)
Total Length (1)*	8.55 (1.14) 6.1–11.0	7.68 (0.85) 5.7–9.4	7.63 (0.78) 5.9–9.6	8.09 (0.84) 5.6–10.1
Carapace Length (2)	3.88 (0.34) 3.0–4.5	3.53 (0.33) 2.6–4.1	3.71 (0.29) 3.0–4.3	3.94 (0.34) 2.7–4.6
First Antenna Length (3)	3.16 (0.13) 2.8–3.4	3.58 (0.16) 3.1–4.0	2.94 (0.17) 2.5–4.1	3.25 (0.21) 2.5–3.6
First Antenna Segment 1 (4)	1.00 (0.09) 0.8–1.2	1.15 (0.11) 0.8–1.4	0.94 (0.10) 0.7–1.3	1.08 (0.10) 0.8–1.4
First Antenna Segment 2 (5)	1.40 (0.08) 1.2–1.6	1.57 (0.10) 1.3–1.8	1.24 (0.08) 1.0–1.4	1.39 (0.11) 1.0–1.6
First Antenna Segment 3 (6)	0.79 (0.06) 0.6–0.9	0.88 (0.07) 0.7–1.0	0.77 (0.07) 0.6–0.9	0.82 (0.08) 0.5–1.0
Diameter of Second Antenna (7)	0.54 (0.09) 0.3–0.7	0.52 (0.06) 0.3–0.7	0.50 (0.08) 0.3–0.6	0.52 (0.09) 0.2–0.7
Second Antenna Endopod Length (8)	1.90 (0.09) 1.5–2.1	2.22 (0.11) 2.0–2.5	1.78 (0.12) 1.6–2.8	2.03 (0.13) 1.7–2.5
Second Antenna Exopod Length (9)	2.07 (0.15) 1.6–2.5	2.48 (0.20) 1.9–3.0	1.99 (0.13) 1.6–2.3	2.20 (0.19) 1.4–2.7
Second Antenna Endopod Segment 1 Length (10)	0.45 (0.06) 0.3–0.8	0.66 (0.08) 0.5–0.8	0.40 (0.05) 0.3–0.5	0.53 (0.06) 0.4–0.7
Second Antenna Endopod Segment 2 Length (11)	0.91 (0.07) 0.7–1.0	1.00 (0.07) 0.8–1.1	0.89 (0.07) 0.7–1.0	1.00 (0.08) 0.7–1.2
Second Antenna Endopod Segment 3 Length (12)	0.55 (0.06) 0.4–0.6	0.58 (0.05) 0.5–0.7	0.50 (0.05) 0.4–0.6	0.53 (0.06) 0.3–0.7
Abdomen Diameter (13)	0.90 (0.16) 0.5–1.2	0.87 (0.12) 0.6–1.1	0.84 (0.13) 0.5–1.2	0.95 (0.16) 0.3–1.3
First Antenna Diameter (14)	0.52 (0.08) 0.2–0.7	0.45 (0.07) 0.2–0.6	0.44 (0.07) 0.3–0.6	0.48 (0.10) 0.1–1.2

* Morphological character number—Figure 3 and text.
Measurements are in ocular divisions (1 O.D. = 0.118 mm).

from the normal deep blue-green to grey, within minutes of capture. Enomoto (1971) reported a similar occurrence for *P. semisulcatus* in Kuwait. This color change was often accompanied by partial spawnings of non-viable eggs. This phenomenon was observed over four cruises in the period November 1980 to May 1981 off Cairns. Viable eggs were finally obtained following shipment of ripe females to our laboratory and inducement by eyestalk ablation.

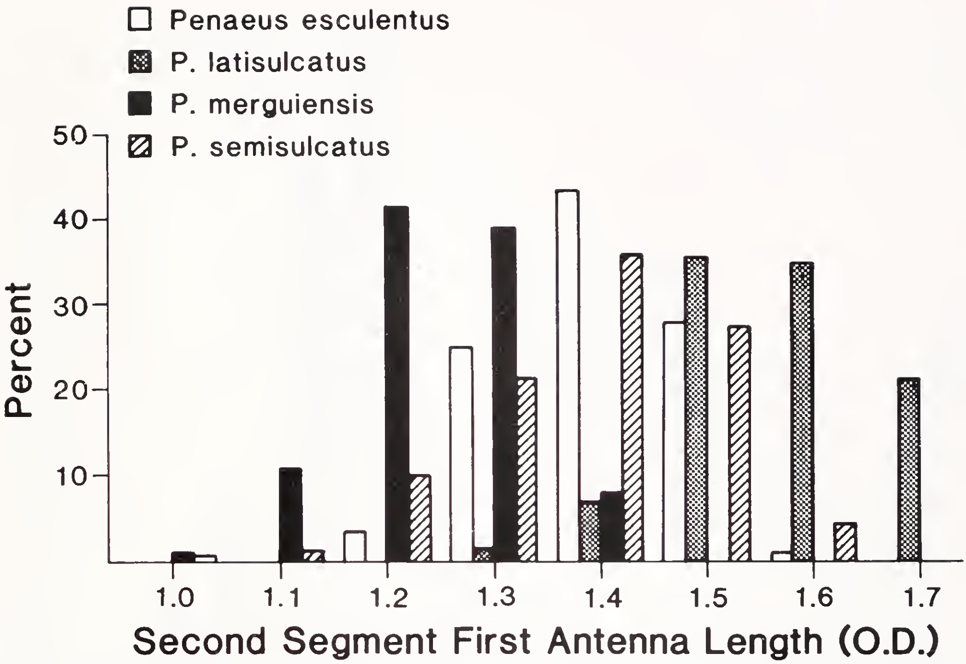

FIGURE 5. Frequency distribution of the length of the second segment of the first antenna of first zoeal instar for four species: *Penaeus esculentus; P. latisulcatus; P. merguiensis;* and *P. semisulcatus.* Ocular division (O.D.) = 0.118 mm.

Though *P. latisulcatus* occurs in the Gulf of Carpentaria we have never caught a suitably ripe female. The larvae in our reference collection were obtained from a commercial prawn farm in Port Broughton, South Australia, on two separate occasions.

Our reference collection (Table I) now includes 15 (*M. bennettae* does not occur in the Gulf) of the 37 species of penaeids reported from the Gulf of Carpentaria and surrounding waters (Dall, pers. comm.). More importantly, we have complete larval series for the four numerically dominant commercial species in the genus *Penaeus: P. esculentus, P. merguiensis, P. latisulcatus,* and *P. semisulcatus.* Four other species (*P. indicus, P. japonicus, P. longistylus,* and *P. monodon*) are known to occur in the Gulf, but at present are numerically insignificant.

Larval morphology

An initial examination of the morphological data for the four *Penaeus* species revealed that, although there was considerable overlap, most characters showed differences between the species (Table II). The range in size and the overlap in distributions between species for the second segment of the first antenna is an example (Fig. 5). When larvae sampled within a few hours of the molt to 1st zoea are compared with larvae sampled toward the end of that instar, it is apparent that some of the characters (*e.g.*, total length, Fig. 6A) undergo considerable change during the intra-molt period. However, other characters (*e.g.*, length of first antenna, Fig. 6B) exhibit less of this type of variation. Therefore, any method of identifying early larval penaeids must allow for considerable variation in morphology.

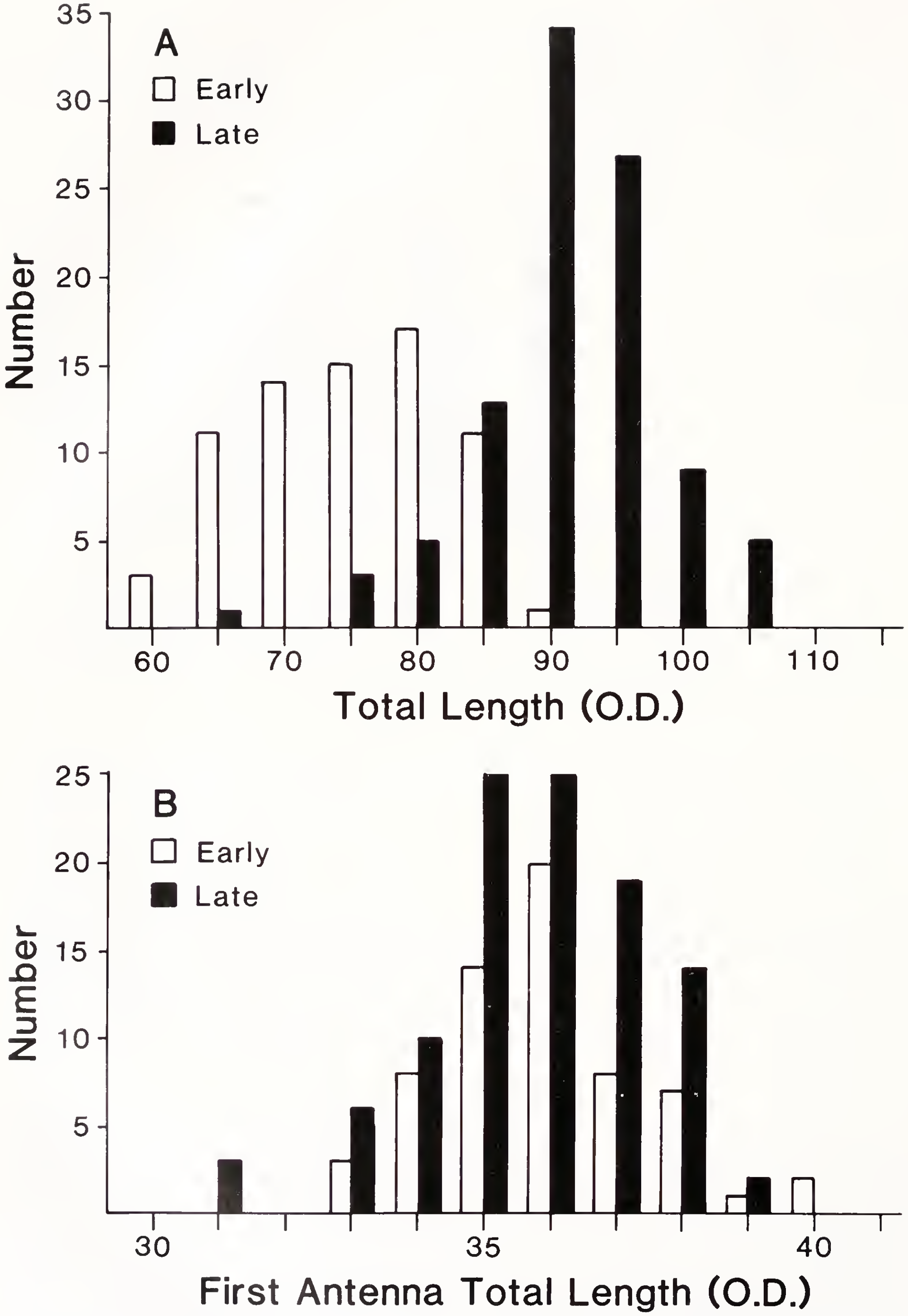

FIGURE 6. A. Frequency distributions of total length indicating growth of this character during the first zoeal instar of *Penaeus latisulcatus*. B. Absence of growth in the length of the first antenna during the first zoeal instar of *P. latisulcatus*. Ocular division (O.D.) = 0.118 mm.

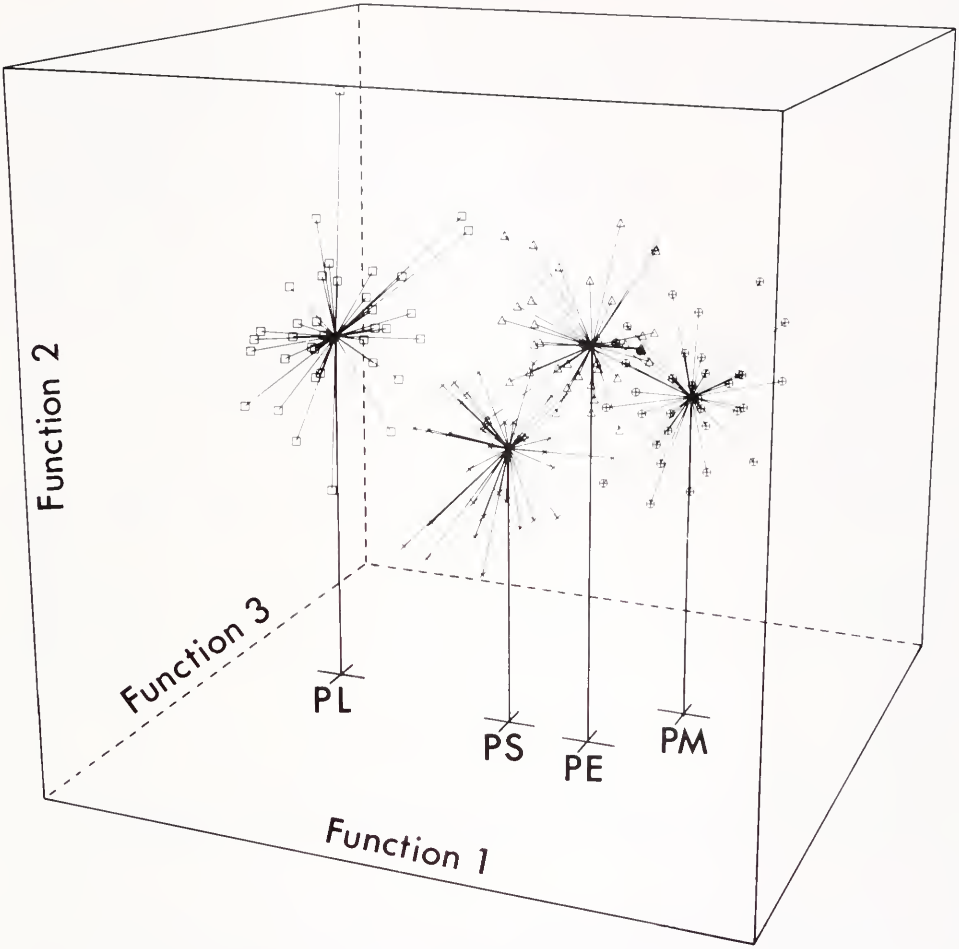

FIGURE 7. Three dimensional separation of four species: *Penaeus esculentus* (PE); *P. latisulcatus* (PL); *P. merguiensis* (PM); *P. semisulcatus* (PS) using three discriminant functions.

Larval identification

A stepwise discriminant analysis applied to the data for the four *Penaeus* species produced three discriminant functions which, when used to classify the reference collection larvae (as if their identity was unknown) resulted in 87.3% overall correct classification. Characters 6 and 7 (Fig. 3) however, did not contribute significantly to this separation, and the information provided by characters 4 and 13, although significant, was small. Characters 3 and 8, although providing useful information, were not necessary since the same information was provided by 4, 5 and 6 in the case of 3, and by 10, 11 and 12 in the case of 8. Therefore a second discriminant analysis was performed with characters 3, 4, 6, 7, 8 and 13 excluded. The resulting 8 characters produced 3 discriminant functions which correctly classified 85.4% of the larvae. The amount of three-dimensional separation achieved according to their score on each of the 3 discriminant functions is shown in Figure 7.

TABLE III

Effect of criterion value (see text for definition) on accuracy of larval identification of four species of Penaeus

	Criterion value							
	1.000	1.001	1.002	1.003	1.004	1.005	1.006	1.007
P. esculentus								
Percent correctly identified*	80.8	83.6	85.4	86.8	88.9	91.1	92.2	93.6
Percent unidentifiable**	0.0	5.6	10.6	15.5	21.7	30.4	36.6	41.6
P. latisulcatus								
Percent correctly identified	95.6	95.6	95.6	97.0	97.0	97.7	97.7	98.4
Percent unidentifiable	0.0	0.0	0.0	1.5	2.2	3.7	3.7	4.4
P. merguiensis								
Percent correctly identified	89.1	91.3	92.1	94.7	95.6	97.7	98.8	98.8
Percent unidentifiable	0.0	5.5	8.2	13.6	17.3	21.8	23.6	26.4
P. semisulcatus								
Percent correctly identified	81.5	83.7	85.0	86.1	88.9	89.3	89.2	90.8
Percent unidentifiable	0.0	4.8	8.5	12.2	16.7	20.4	28.2	31.5
Overall								
Percent correctly identified	85.4	87.4	88.6	90.1	91.8	93.0	93.6	94.7
Percent unidentifiable	0.0	4.1	7.2	11.1	15.0	19.6	24.5	27.6

* Percent of the number of larvae that were above the criterion level and were correctly identified.

** Percent of the number of larvae below the criterion level, and therefore identification was uncertain (see text).

The SPSS Discriminant program provides an easy method for identifying unknown larvae. The discriminant functions are used to generate a set of classification functions. In this case there are four classification functions, one corresponding to each of the four *Penaeus* species. An unknown larva can be identified by using it's eight measurements to calculate four scores, one for each of the four functions. The unknown is then classified according to the highest score. In this study, in order to increase the accuracy of identification, for each unknown the ratio between the two highest classification scores was examined. If this ratio (= criterion) fell below a certain level the identification reverted to unidentified, on the basis that the initial identification was relatively uncertain. The effect of varying this criterion value when the larvae of the reference collection were identified in this way is shown in Table III. The proportion of correct identifications increased from 85.4%, when all identifications were accepted, to 94.7% when the test was severe enough to classify 27.6% of doubtful larvae as unidentified. At all criterion levels *P. latisulcatus* was readily separated from the other three species, having a high percentage of correct classifications and the lowest proportion remaining unidentified. On the other hand, the two tiger prawns *P. esculentus* and *P. semisulcatus* were the most similar: adults of these species are also difficult to distinguish. As a compromise between increasing the proportion of correct identifications, and reducing the amount of lost information due to larvae being classified as unknown, an operational criterion value of 1.003 was chosen. This produced overall 90.1% correct identifications, while 11.1%

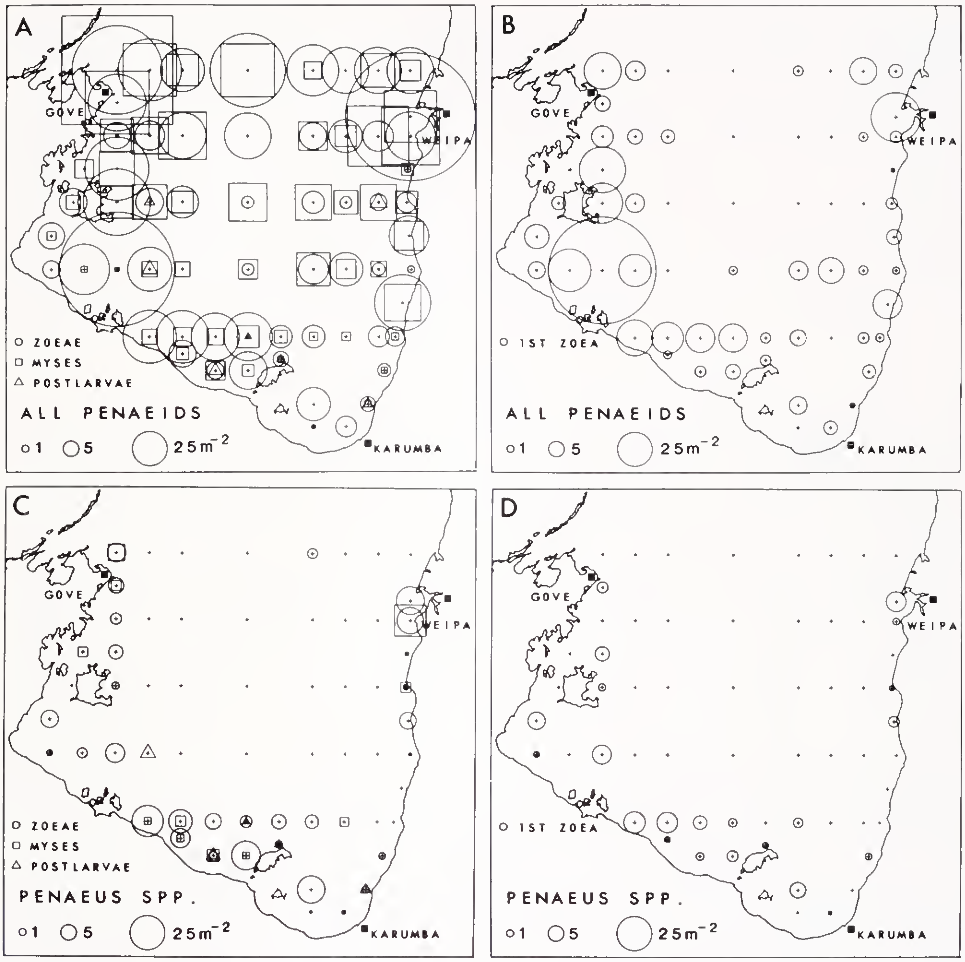

FIGURE 8. Larval distribution and abundance in the Gulf of Carpentaria in March 1977. A. Three zoeal and three mysis larval substages and postlarvae of all species of penaeids. B. First zoeal larval substage of all species of penaeids. C. Three zoeal and three mysis larval substages and postlarvae of all species in the genus *Penaeus*. D. First zoeal larval substage of all species in the genus *Penaeus*.

remained unidentified. At this level the two species of tiger prawn were accurately identified more than 86% of the time.

An interactive computer program was written to identify larvae routinely in this way. A technician, using a computer terminal, is prompted for values for the eight measurements, and these are queried by the program if they fall outside the normal range. Classification scores are calculated, and the ratio between the two highest scores is compared with a criterion of 1.003; the resulting identification is then reported to the operator. Identifications, together with all character measurements, are stored on floppy discs for later use.

Larval distribution and abundance

Penaeid larvae, potentially from almost 40 species, were found in most samples taken in the Gulf of Carpentaria in March 1977 (Fig. 8A). First zoea larvae appear

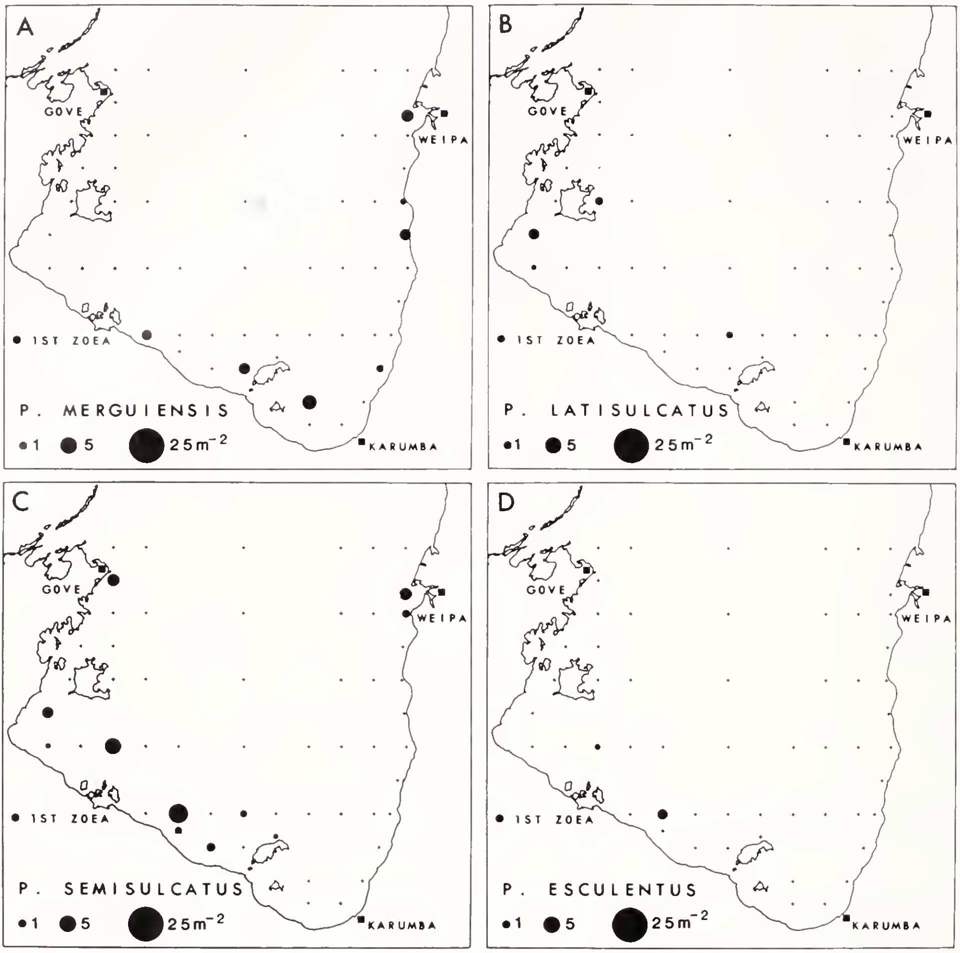

FIGURE 9. Distribution and abundance of the first zoeal instar of four species of *Penaeus* in the Gulf of Carpentaria, Australia in March 1977. Identification of species by discriminant function method, using criterion = 1.003. See text for details of method. A. *Penaeus merguiensis*. B. *P. latisulcatus*. C. *P. semisulcatus*. D. *P. esculentus*.

approximately 48 hours after spawning and their presence is a good indication of spawning activity; for this reason it seemed unnecessary to attempt the difficult task of sampling nauplii quantitatively and identifying them for the sake of marginally higher resolution of spawning time and location. The distribution of first zoeae indicated that penaeid spawning was widespread and abundant (Fig. 8B).

Penaeus larvae and postlarvae were restricted to a relatively narrow coastal band (Fig. 8C). Thus the large numbers of early penaeid zoeae found in much of the Gulf of Carpentaria (Fig. 8B) were not *Penaeus* species (Fig. 8D).

With application of the discriminant analysis, specific resolution of the first zoeal distributions was obtained (Fig. 9). The distribution of *P. merguiensis* larvae (Fig. 9A) was discontinuous and restricted to three areas of the Gulf; Albatross Bay, off Weipa in the north eastern Gulf; along the middle eastern side of the Gulf; and the southern Gulf, both east and west of Mornington Island. Early larvae of *P. latisul-*

catus (Fig. 9B) were absent from the eastern side of the Gulf and were most abundant around Groote Eylandt. First zoeae of *P. semisulcatus* (Fig. 9C) were the most widespread of the four species as spawning occurred in Albatross Bay on the north eastern side of the Gulf and over an extended area on the western side, from the Gove peninsula, south of Groote Eylandt, down to west of Mornington Island. *Penaeus esculentus*, though very abundant as adult shrimp at this time, did not appear to be spawning (Fig. 9D).

The decreased number of larvae represented in Figure 9, compared with Figure 8D, was due either to the presence of larvae of species other than the four revealed by the identification procedure, or to larvae of those four species, but with morphology falling outside the acceptable limits set by our criterion values.

DISCUSSION

In temperate penaeid fisheries, where diversity is relatively low and there may be temporal and spatial separation of reproductive activity by species, larvae in the plankton can be 'identified' on a probability basis with some assurance, given knowledge of adult distribution, abundance and reproductive state (Munro *et al.*, 1968; Roessler *et al.*, 1969; Jones *et al.*, 1970). In tropical penaeid fisheries which are characterized by highly diverse genus and species assemblages, there may be little spatial or temporal separation of their prolonged reproductive activity. This is particularly true in the Indo-West Pacific, including the Gulf of Carpentaria. Here it is not possible to guess the identity of larvae by examining the temporo-spatial distribution of spawning adults, and the larvae must be identified with a robust taxonomic method.

Establishing a reference collection of penaeid larvae has been the first step in enabling identification of larvae from our plankton collections. The shipboard rearing technique we developed was extremely useful, allowing us to spawn adults and rear larvae in remote areas with no laboratory facilities. Shipment of ovigerous females or eggs from these locations would not have been practicable.

Given the high degree of variation in morphology within species, as well as that due to growth within instars, the few larval descriptions in the literature were of little value because they were based on small numbers of specimens and did not report characters which had taxonomic value at the species level. A collection with large numbers of larvae, sampled at frequent intervals from diverse sources, was necessary.

Even with a large amount of larval material at hand however, it became obvious that simple, single character comparisons could not be used for separating penaeid larvae because of the large degree of overlap in both merisitic and morphometric characters. A technique had to be found that was practicable for screening large numbers of larvae from field collections. Characters were useful only if they could be measured with a minimum of handling and no dissection. Discriminant analysis, a multivariate technique developed by Fisher (1936), sums the information provided by a number of quantitative values, each of which shows incomplete, but significant differences between species. Although this is the first time the technique has been applied to larval crustacean taxonomy, it has been applied to a variety of taxonomic problems, for example: Africanized honeybee hybrids (Daly and Balling, 1978); pink salmon (Pearson, 1964); human crania (Giles and Elliot, 1962, 1963); caddis larvae (Buholzer, 1977); nematode eggs (Lysek *et al.*, 1975); and adult American lobster populations (Saila and Flowers, 1969).

There are several areas of penaeid larval identification requiring further development. The first is to apply the technique to later zoeal instars and later larval stages. This involves morphological character assessment on a instar by instar basis. Work is proceeding on experimental larval rearing studies to test the validity of the numerical taxonomic technique, bearing in mind the morphological lability seen in the early larvae. We are also increasing the number of species in our reference collection, not only so we can identify the still unidentified larvae in our samples, but also to see if the technique can be used in other geographic species complexes or broadened to the whole genus *Penaeus.* Work on the genus *Metapenaeus,* which also supports important fisheries, is underway. Finally, having developed a complex interactive procedure of larval examination and identification there is some evidence that a skilled operator, with some experience, can identify some species based on general shape and other subtle characters that are very hard to quantify. If these subjective criteria can be made more objective and repeatable between observers, the time, expense and technology required for larval identification should be lessened and the application broadened.

Our progress with larval identification has begun to resolve the confusion regarding penaeid larval distribution and abundance in the Gulf of Carpentaria. As the rest of the larvae from our survey cruises are analyzed, a better Gulf-wide picture of temporal and spatial reproductive dynamics, for the four most important commercial species at least, will be possible. As the later larval stages are incorporated into the identification scheme, factors affecting larval mortality during this short and probably critical planktonic period can be assessed. Finally, hypotheses and mechanisms regarding postlarval recruitment (Staples, 1979) and larval advection (Rothlisberg, 1982; Rothlisberg *et al.,* in press) can be tested in the field.

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β-1,3 GLUCAN ACTIVATION OF CRUSTACEAN HEMOCYTES *IN VITRO* AND *IN VIVO*

VALERIE J. SMITH[1] AND KENNETH SÖDERHÄLL[2]

[1] *University Marine Biological Station, Millport, Isle of Cumbrae, Scotland KA28 OEG, and* [2] *Institute of Physiological Botany, University of Uppsala, Box 540, 751 21, Uppsala, Sweden*

ABSTRACT

The effects of β-1,3 glucans on the hemocytes of the freshwater crayfish, *Astacus astacus,* and the shore crab, *Carcinus maenas,* were studied *in vitro* and *in vivo* to determine the role of the prophenoloxidase activating system, in the cellular defense reactions of crustaceans.

In vitro, phagocytosis of the bacterium, *Moraxella* sp. was significantly raised by addition of laminarin, a β-1,3 glucan, simultaneously with the test particles to the hemocytes in monolayer cultures. Both the proportion of cells ingesting one or more bacterial particles and the number of bacteria taken up by individual cells were increased, and the responses were found to be time dependent and dose related. Glucose, dextran, cellulose, and chitin had no stimulatory influence on the cells, and the agglutination of erythrocytes by crab hemocytes or serum was unchanged by glucan incubation. Examination of the monolayers under phase contrast microscopy, revealed that the glucans induced degranulation and occasionally lysis in the crayfish hemocytes, and vacuolation in the crab hemocytes.

In vivo, injection of β-1,3 glucans (0.2 mg laminaran/ml hemolymph) into the hemocoel of *A. astacus* or *C. maenas* caused a rapid, marked reduction in the number of circulating hemocytes, indicating that a cellular defense reaction was initiated. Since prophenoloxidase in the hemocytes is specifically activated by β-1,3 glucans, and in the activated form phenoloxidase is "sticky," it is suggested that certain proteins of the prophenoloxidase activating system may serve as opsonins, and possibly constitute an important recognition mechanism in crustaceans.

INTRODUCTION

Lacking immunoglobulins, invertebrates are potentially useful models for analyses of the non-specific immune processes, such as Ig-independent phagocytosis and cytotoxicity. But, despite the numerous observations of the various humoral and cellular defense reactions in different species (Ratcliffe and Rowley, 1979), the molecular basis for the recognition of non-self materials is still not fully understood. Recently, however, β-1,3 glucans, carbohydrates from fungal cell walls which have a potent stimulatory action on the reticuloendothelial system of mammals (Kokoshis *et al.,* 1978; Di Luzio, 1979), have been found to elicit melanization (Unestam and Söderhäll, 1977; Söderhäll and Unestam, 1979) and coagulation reactions in the hemolymph of arthropods (Kakinuma *et al.,* 1981; Morita *et al.,* 1981; Söderhäll, 1981). In crustaceans and insects, the glucans exert their effect by specifically activating prophenoloxidase, a proenzyme involved in melanin synthesis, in the hemocytes or plasma, through a complex enzyme cascade including at least one serine protease (Ashida, 1981; Söderhäll, 1981; Söderhäll, 1983). In arthropods, melanin

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deposition frequently accompanies the host cellular responses to wounding, foreign implants, parasites or invading micro-organisms (Salt, 1970; Ratcliffe and Rowley, 1979) and, in insects (Salt, 1970; Vey, 1979), phenoloxidase appears to be associated with host resistance. Experiments were therefore carried out to determine the effect of β-1,3 glucans on the hemocytes and to examine the role of prophenoloxidase in hemocyte activity. Using the freshwater crayfish, *Astacus astacus,* for which the prophenoloxidase activating system has been defined (Söderhäll, 1982, 1983), and the shore crab, *Carcinus maenas,* as convenient hosts, phagocytosis, a phenomenon universal throughout the animal kingdom was studied *in vitro.* The influence of the glucans on the hemocytes *in vivo* and the agglutination of test particles *in vitro* were also investigated since in invertebrates agglutinins are thought to function as recognition factors or opsonins (McKay and Jenkin, 1970; Renwrantz and Cheng, 1977).

MATERIALS AND METHODS

Animals

Specimens of *Astacus astacus* were collected from Lake Vallsjön, Småland, Sweden, and kept as described in Söderhäll and Unestam (1979) *Carcinus maenas* were obtained, with creels, from Balloch Bay, Firth of Clyde, Scotland and housed as previously reported (Smith and Ratcliffe, 1978). Only healthy, intermoult animals were used for experimental purposes.

Culture media

Balanced salt solutions, made up to resemble the ionic composition of *A. astacus* or *C. maenas* hemolymphs were used for the culture of the hemocytes *in vitro* and other experiments. The composition of crayfish saline (CFS) was: 0.2 mM NaCl; 5.4 mM KCl; 10 mM $CaCl_2$; 2.6 mM $MgCl_2$ and 2.0 M $NaHCO_3$, pH 6.8; and the composition of *Carcinus* saline (CS) was as given in Smith and Ratcliffe (1978). Preliminary tests confirmed that hemocyte viability, determined by trypan blue exclusion, remained as high as 98% for 6 h at 20°C in these salines.

Glycans

Laminarin (Calbiochem), a β-1,3 glucan, prepared as described in Söderhäll and Unestam (1979), was suspended in sterile CFS or CS at an initial concentration of 1%, dissolved by heating and then diluted to the required strength in CFS and CS. Glucose (Merck), dextran (T40, Pharmacia Uppsala), cellulose (Avicel, OP 300, Kebo, Stockholm) and chitin (prepared as described in Söderhäll and Unestam (1979) were similarly dissolved but to concentrations of 0.2%. In addition, a crude β-1,3 glucan fraction (Zs) was also obtained from the supernatant of a 1% zymosan (Sigma Chemical Co., Kingston-upon-Thames, Surrey) suspension (Söderhäll and Unestam, 1979) in double distilled water, and was used in the prophenoloxidase estimation assays for *C. maenas.*

Bacteria

The gram negative bacterium, Moraxella sp. (NCMB 308) was grown and prepared as described in Smith and Ratcliffe (1978). The washed bacteria were suspended in CFS or CS at a concentration of 2.0×10^7 ml^{-1}, before dilution with an equal volume of either the desired glycan solution or the appropriate culture saline.

Preparation and treatment of monolayers

Crayfish hemocyte monolayers were prepared by mixing *ca.* 200 μl of hemolymph, bled from the abdominal hemocoel with an 18 g needle, to 100 μl of CFS, containing cysteine (Sigma) (15 mg ml^{-1}) as an anticoagulant, on clean pyrogen-free coverslips. The coverslips, in sterile, plastic multiwell trays, were left to stand at 20°C for 15 min to allow the cells to settle and attach to the glass surfaces, before being washed with two 2.5 ml volumes of sterile, filtered CFS. Similar monolayers were prepared for *C. maenas* according to the method described by Smith and Ratcliffe (1978).

Initially, to observe the effects of β-1,3 glucans on crayfish and crab hemocytes *in vitro*, freshly prepared monolayers were overlaid with 100 μl of 0.1% laminarin or, in the case of controls, with 100 μl of CFS or CS. Following incubation at 20°C for 1 h, the hemocytes were fixed in 2.5% glutaraldehyde (Agar Aids, Bishop Stortford, Herts) in CFS or CS (pH 6.8 or 7.4) and examined under phase contrast optics of a Leitz Dialux 20 microscope.

Next, to examine the influence of glycans on the phagocytic capacity of *A. astacus* and *C. maenas* hemocytes *in vitro*, two series of monolayers were prepared for each animal. One series was given 100 μl doses of *Moraxella* sp. in 0.1% laminarin, while the second received 100 μl of *Moraxella* in CFS of CS. For comparison, additional monolayers were inoculated with 100 μl of bacteria suspended in 0.1% glucose or the appropriate culture saline, and, to provide some information as to the specificity of the cellular responses to the glycans, hemocyte monolayers, prepared from three crayfish, were challenged with 100 μl of bacteria in 0.1% dextran, cellulose or chitin, and, for the controls, crayfish saline.

After inoculation, the coverslips were placed in a moist chamber and incubated on a rocking platform for 2 h at 20°C. The cultures were then removed, rinsed twice with CFS or CS and fixed in 2.5% glutaraldehyde (15 min) before examination under phase contrast optics.

In a separate experiment, designed to evaluate the dose response, bacterial suspensions made up in 0.01% or 0.001% laminarin were added, in 100 μl doses, to preformed crayfish or crab monolayers, and the cultures incubated, rinsed, fixed and examined as above. Control monolayers, prepared from the same animals, were similarly treated but with bacterial suspensions made up in CFS or CS only.

Finally, to investigate the kinetics of the cellular reaction, hemocyte cultures of *A. astacus* or *C. maenas* were preincubated with laminarin (100 μl of a 0.1% solution) or, in the controls, with saline, for 1 h at 20°C prior to addition of 100 μl of the bacterial suspension. The monolayers were incubated, rinsed and fixed as described above.

Quantification of phagocytosis and analysis of results

Phagocytosis of bacteria in the experimental, *i.e.* glycan treated, and control, saline treated, monolayers was quantified by scoring the number of cells containing one or more intracellular particles (the percentage phagocytosis), and by recording the number of bacteria ingested per 100 hemocytes. Intracellular bacteria were distinguished from extracellular, adherent forms using the criteria described by Smith and Ratcliffe (1978). A minimum of 400 cells was observed on randomly chosen areas from each of three coverslips for each treatment and every animal, and, to eliminate the bias inherent in this method of assessment, all counts were made blind. Unless otherwise stated, groups of 5–10 crayfish or crabs were used in each experiment.

From the triplicate coverslips, the median values for the percentage phagocytosis and the number of ingested particles were recorded for the experimental and control treatment of every animal. Differences in the rate of uptake between glycan and saline incubated monolayers were analysed statistically with the Walsh test for related samples (two tailed), and differences in the rates of uptake between glucan concentrations were analysed with the Mann Whitney U test (one tailed) (Siegel, 1956). The specified level of significance for both tests was ≤ 0.05.

To emphasize the change in the cellular responses to the bacteria induced by glycan treatment, the results were also expressed as the enhancement index (EI). This was derived for each animal from the formula:

$$\frac{\text{Rate of uptake in experimental (glycan treated) cultures}}{\text{Rate of uptake in control (saline treated) cultures}} \times 100$$

Enhanced uptake is thus shown by EI values greater than 100, whereas suppression of phagocytosis is indicated by values for the EI below 100.

Preparation of crab hemocyte lysate supernatant (HLS) and assay of phenoloxidase activity

Although in crayfish β-1,3 glucans are known to activate prophenoloxidase within the hemocytes (Söderhäll and Unestam, 1979; Söderhäll, 1981), equivalent events have not been demonstrated for *C. maenas*. In this investigation, therefore, hemocyte lysates were prepared from crab hemolymph, activated with β-1,3 glucans and then assayed for phenoloxidase and protease activity.

For the HLS, hemocytes were harvested from six crabs by withdrawing 2.25 ml of hemolymph from each crab, into a 5 ml syringe containing 2.5 ml of ice-cold cacodylate buffer (10 m*M* sodium cacodylate; 5 m*M* $CaCl_2$; 0.25 *M* sucrose and 0.1 *M* sodium citrate), pH 7.0. The samples were pooled, centrifuged at 800 g for 10 min and the pellet washed once with 50 ml of citrate-depleted cacodylate buffer, pH 7.0, at 4°C. The hemocyte pellet was then homogenized in 4.0 ml of cacodylate: $CaCl_2$ buffer (10 m*M* sodium cacodylate; 5 m*M* $CaCl_2$) pH 7.0, and the supernatant remaining after centrifugation at 40,000 g for 30 min (4°C) was used as the enzyme source. For the hemagglutination assays (see below), the hemocyte pellet was homogenized in 4.0 ml of CS at 4°C before centrifugation at $40{,}000 \times g$ (10 min) and storage at −4°C.

Phenoloxidase activity in glucan or saline incubated HLS was determined using L-dihydroxyphenylalanine (L-dopa) as substrate (Söderhäll, 1981). In the experimental tubes 400 μl of crab HLS was preactivated with 200 μl of 0.1% laminarin or Zs, and 200 μl of 200 m*M* $MgCl_2$ for 1 h at 20°C; 200 μl of this reaction mixture was then added to 200 μl of L-dopa (4 g l^{-1}) with 400 μl of cacodylate buffer (10 m*M* sodium cacodylate; 5 m*M* $CaCl_2$) pH 7.0, and after 5 min at 20°C, the absorbance was read at 480 nm. Control mixtures, in which distilled water or CS was substituted for the glucans in the activation step, were run parallel to the experimentals, and for all samples, phenoloxidase activity was expressed in units: one unit representing the amount of enzyme required to produce an increase in absorbance of 0.001 min^{-1}. Preliminary tests confirmed that activity was proportional to enzyme concentration and linear with respect to time.

Assay of HLS protease activity

Protease activity was assayed using the synthetic chromogenic peptide, Bz-Ile-Glu-(γ-O-Piperidyl)-Gly-Arg-PNA-HCl (AB Kabi Peptide Research, Mölndal, Sweden), as substrate (Söderhäll, 1983). HLS was first activated with laminarin or Zs as above, and 200 μl of the reaction mixure was incubated with μl of 1.5 m*M* chromogenic peptide for 30 min at 20°C. The reaction was terminated by addition of 100 μl of 50% acetic acid, and the released p-nitroaniline measured spectrophotometrically at 405 nm. Enzyme activity was expressed as the rate of change in absorbance at this wavelength.

Protein determination

The protein content of crab HLS was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin for the standards.

All biochemical measurements were repeated at least three times.

Titration of hemagglutinins

Alsevers stored sheep or horse erythrocytes (Gibco Biocult, Renfrew, Scotland) and fresh, citrated mouse or human type A blood samples were used to examine the effect of β-1,3 glucans on the agglutinating property of crustacean serum or HLS. Before use, the erythrocytes were washed three times in phosphate buffered saline pH 6.8 (Oxoid Ltd., Basingstoke, Hants) and resuspended in culture saline at a concentration of 4% v/v.

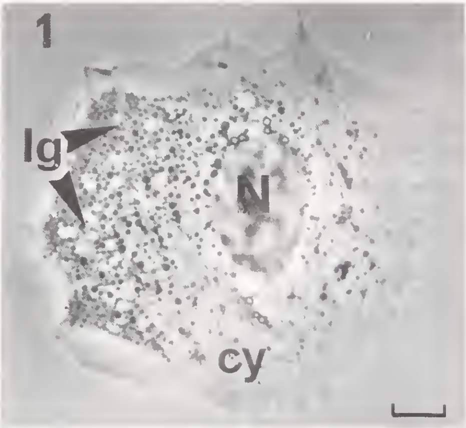
Crab serum was prepared from 6–8 animals as described in Smith and Ratcliffe (1978), and *C. maenas* HLS was made, in CS, as above. Aliquots of 50 μl of serum or HLS were serially diluted twofold in CS in microtitre trays (Sterilin Ltd), and to one series of wells was added 25 μl of 0.2% laminarin while a second received 25 μl of CS. For the controls, 25 μl of 0.2% laminarin or CS was mixed with 25 μl of crab saline, and finally, 25 μl of the appropriate washed erythrocyte suspension was pipetted into each well. The trays were incubated for 2 h at 20°C and the titres were recorded as the last serum or HLS dilution showing visible agglutination. Comparable measurements were not made on crayfish serum or HLS as the results of these experiments for *C. maenas* failed to reveal any changes in HA activity with glucan treatment (see Results below).

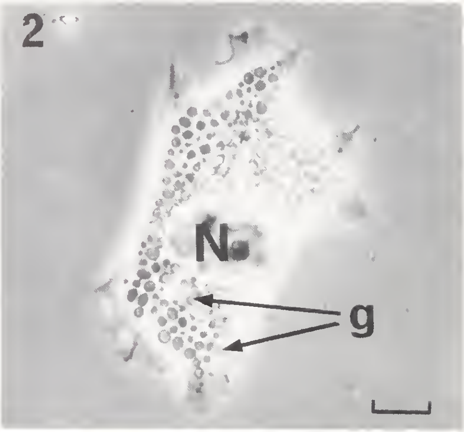
Effect of β-1,3 glucans on A. astacus *and* C. maenas *hemocytes* in vivo

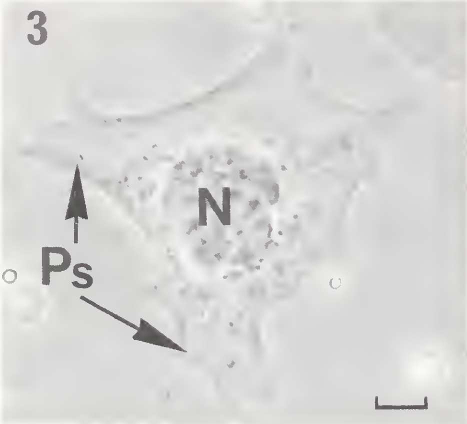
As further evidence of the activating effect of β-1,3 glucans on crayfish and crab hemocytes observed *in vitro* (see Results below), preliminary *in vivo* investigations were also carried out. For these, 0.1% laminarin in CFS or CS was injected, using 28 g needles, into the unsclerotized membrane at the base of the fourth pereiopod of crayfish (measuring 70–90 mm in length) or crabs (measuring 68–72 mm across the carapace).

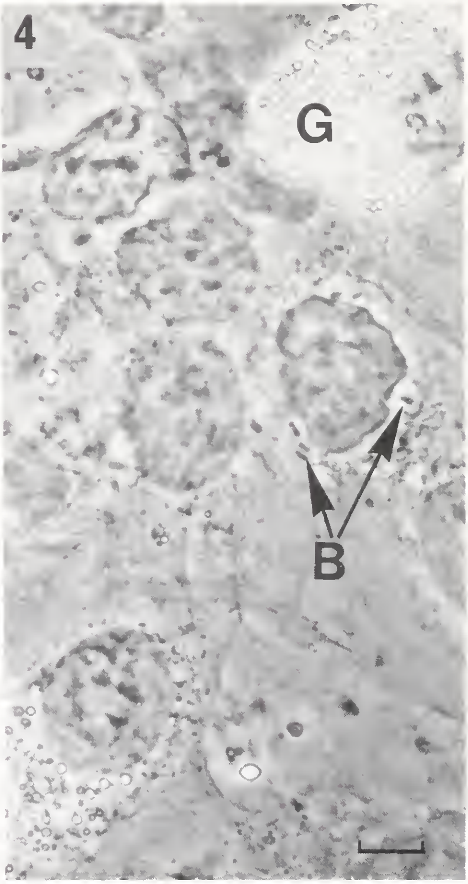
To compensate for the difference in the hemolymph volumes between *A. astacus* and *C. maenas*, the size of the inoculum was adjusted so that each animal received 0.2 mg laminarin per milliliter of hemolymph. Control animals were similarly inoculated but with CFS or CS only.

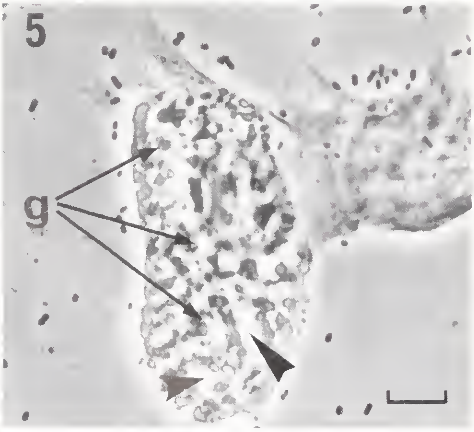
The crayfish were incubated in clean, aerated tap water at 13°C, and the crabs in filtered, circulating sea water at 15°C, for 30 min, 3 h or 24 h. At each time interval, groups of 5–8 animals were removed and a known volume of hemolymph withdrawn from the abdominal hemocoel, as above, into an equal volume of 2.5% glutaraldehyde. The numbers of hemocytes present in the hemolymph samples were ascertained using improved Neubauer hemocytometers, and the counts were expressed as the total hemocyte number (THC) per milliliter of hemolymph. For the











FIGURES 1–3. Hemocytes of *A. astacus* incubated for 1 h *in vitro* with CFS. Phase contrast optics.

FIGURE 1. Well spread, intact, semi-granular cell. Note the central nucleus (N) and numerous, small intracellular inclusions (Ig) in the cytoplasm (cy). Scale bar 10 μm.

FIGURE 2. Granular hemocyte containing many large, highly refractile granules (g). Nucleus (N). Scale bar 10 μm.

time zero values, similar hemolymph samples were taken from groups of untreated crayfish or crabs and the THC determined as above.

Differences in the THC between untreated, saline injected and glucan injected crayfish or crabs were analysed statistically using the Mann-Whitney U test (one tailed) (Siegel, 1956). The specified level of significance was $P \leq 0.01$.

RESULTS

Effects of β-1,3 glucans on the hemocytes of A. astacus *and* C. maenas in vitro

Monolayers of *A. astacus* were seen to contain three morphologically distinct hemocyte types (Figs. 1, 2, 3). The most abundant were the semi-granular hemocytes (Fig. 1), which comprised *ca.* 50% of the cells on the coverslips, and were usually flattened and well spread, with a central nucleus and a variable number of small (*ca.* 1 μm diameter) intracellular inclusions. The second cell type, the granular cells, was similar to the semi-granular hemocytes in size and shape, but always enclosed numerous, large (*ca.* 1–3 μm diameter), highly refractive granules (Fig. 2), and represented *ca.* 30% of the cells in the cultures. The remaining cells in the monolayers were the hyaline amoebocytes (Fig. 3) and these cells were distinguished from the others by the lack of large distinct cytoplasmic inclusions and the formation of long pseudopodial extensions during attachment to the coverslip surface. The monolayers of *C. maenas* were composed of flattened, well spread hyaline cells, as described previously (Smith and Ratcliffe, 1978), and incubation of crayfish or crab cultures in saline for 1 h produced no visible effects on the hemocytes (Figs. 1–4, and 6).

However, in *A. astacus,* treatment with 0.1% laminarin caused marked degranulation with consequent vacuolation in some (*ca.* 70%) of the semi-granular and granular cells (Figs. 5, 7), and occasionally, in the phagocytosis assays, prolonged exposure to the glucans resulted in lysis of *ca.* 10–20% of the cells (Fig. 8).

Granule discharge could not be observed in *C. maenas* hemocytes following glucan treatment since the granular (refractile) cells of this animal are rarely present on the monolayers (Smith and Ratcliffe, 1978). However, addition of 100 μl of L-dopa to the glucan treated monolayers resulted in dopachrome formation, showing that phenoloxidase was present on the coverslips. The hyaline (phagocytic) cells (Smith and Ratcliffe, 1978) of crabs exhibited some vacuolation in response to the glucans (Fig. 9), but the effects were usually less dramatic than in *A. astacus.*

During the phagocytosis studies, neither crayfish nor crab hemocytes showed any morphological changes to 0.1% glucose, and in crayfish, the appearance of the cells in the dextran, cellulose or chitin incubated cultures was similar to those in the saline incubated controls.

Effect of β-1,3 glucans on in vitro *phagocytosis of bacteria*

In vitro, phagocytosis of *Moraxella* sp. by the hemocytes of *A. astacus* and *C. maenas* was enhanced by β-1,3 glucans (Tables I, II). Addition of 0.1% laminarin

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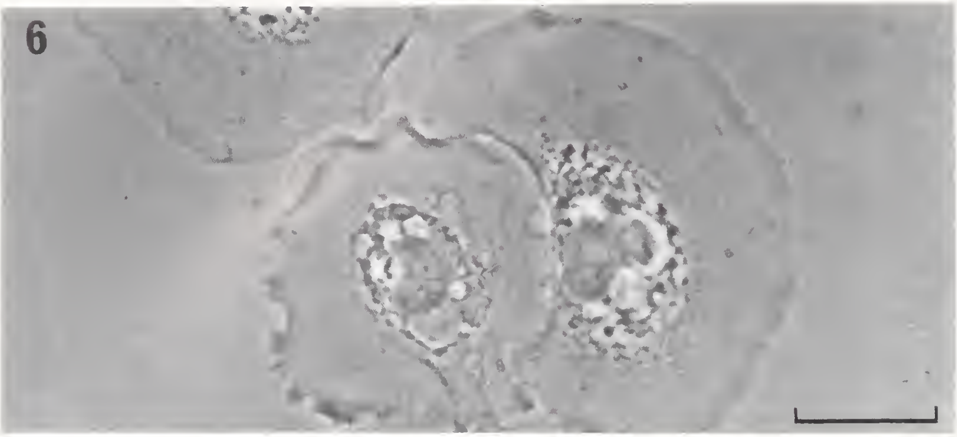
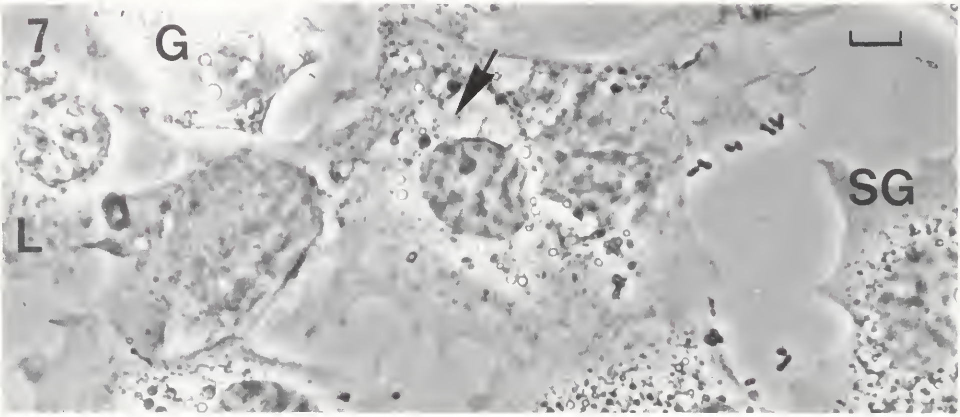
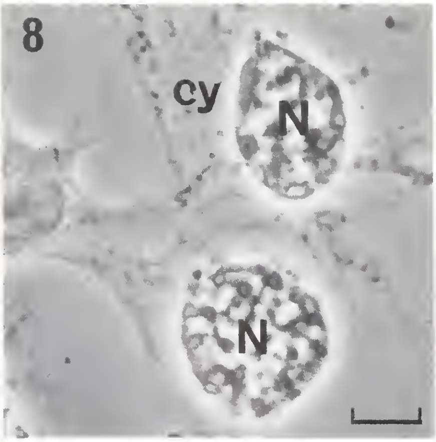
FIGURE 3. Hyaline hemocyte central nucleus (N) and pseudopodial extensions (Ps). Note the absence of large, distinct cytoplasmic inclusions. Scale bar 10 μm.

FIGURE 4. Hemocyte monolayer of *A. astacus* incubated for 2 h with *Moraxella* sp. Most of the cells are of the hyaline type, one of which contains four intracellular bacteria (B). A granular hemocyte (G) is present but none of the cells show signs of damage, vacuolation or lysis. Phase contrast optics. Scale bar 10 μm.

FIGURE 5. Granular hemocyte of *A. astacus* incubated for 1 h in 0.1% laminarin. The cell has partially degranulated, leaving empty spaces in the hemocyte cytoplasm (arrows). Remaining granules (g). Phase contrast. Scale bar 10 μm.







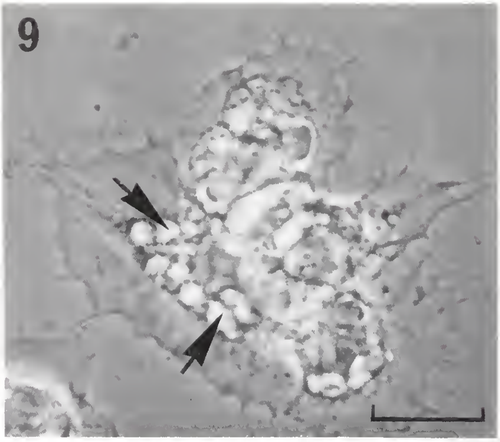


FIGURE 6. Hemocyte monolayer of *C. maenas* incubated for 1 h in CS. All the cells shown are well spread hyaline (phagocytic) types, with no sign of vacuolation or lysis. Phase contrast optics. Scale bar 10 μm.

FIGURE 7. Hemocyte monolayer of *A. astacus*, incubated for 1 h in 0.1% laminarin. Note the vacuoles (arrow) in the central cell, and the remnants of a lysed cell (L). Intact granular (G) and semigranular (SG) cells are also present. Phase contrast. Scale bar 10 μm.

TABLE I

In vitro *phagocytosis of* Moraxella *sp. by the hemocytes of* A. astacus

Treatment of monolayers	Percentage phagocytosis		EI[a]	Number of bacteria/ 100 hemocytes		EI
0.1% laminarin	18.0[b] ± 4.8	$P = 0.008$	328	358 ± 60	$P = 0.008$	160
control[c]	5.6 ± 1.1			223 ± 53		
n = 8[d]						
0.01% laminarin	12.1 ± 4.3	$P = 0.008$	315	446 ± 91	$P = 0.008$	181
control	3.7 ± 1.2			246 ± 68		
n = 5						
0.1% glucose	5.0 ± 2.2	$P > 0.097$	111	267 ± 79	$P > 0.097$	116
control	4.5 ± 1.5			229 ± 56		
n = 6						
0.1% dextran	5.6 ± 3.4	$P > 0.05$	101	226 ± 97	$P > 0.05$	112
control	5.3 ± 2.2			201 ± 27		
n = 3						
0.1% cellulose	8.7 ± 2.4	$P > 0.05$	165	230 ± 89	$P > 0.05$	114
control	5.3 ± 2.2			201 ± 27		
n = 3						
0.1% chitin	5.2 ± 4.1	$P > 0.094$	85	207 ± 71	$P > 0.094$	97
control	5.3 ± 2.2			201 ± 27		
n = 3						
preincubation[e] in 0.1% laminarin	2.3 ± 0.6	$P > 0.094$	85	222 ± 72	$P > 0.094$	94
control	2.6 ± 1.3			235 ± 52		
n = 6						

[a] EI as given in Materials and Methods.
[b] Values given are means ± standard deviation.
[c] Controls were incubated in CFS.
[d] n = number of animals used in experiment.
[e] Preincubation for 1 h at 20°C before challenge with bacteria in CFS.

simultaneously with the bacteria raised the percentage phagocytosis in *A. astacus* from 5.6% to 18.5% (EI = 328) and the number of particles taken up from 223 to 358 (EI = 160) (Table I). Similar results were obtained for *C. maenas* where the EI for the percentage phagocytosis was 303 and the number of bacteria/100 hemocytes was 200 (Table II) (Fig. 10). However, in both *A. astacus* and *C. maenas,* the response was achieved only in freshly prepared cultures overlaid immediately with glucan:bacteria mixtures, and not in those left for longer than 15 min before inoculation (data not presented). Further analyses also revealed that the response was independent of the cell density on the coverslips and the concentration of cysteine used in the monolayer preparatory stage (data not shown).

In contrast to the glucans, glucose (0.1%) was ineffective in promoting uptake, and in *A. astacus* the percentage phagocytosis remained at 5.0% (EI = 111), with

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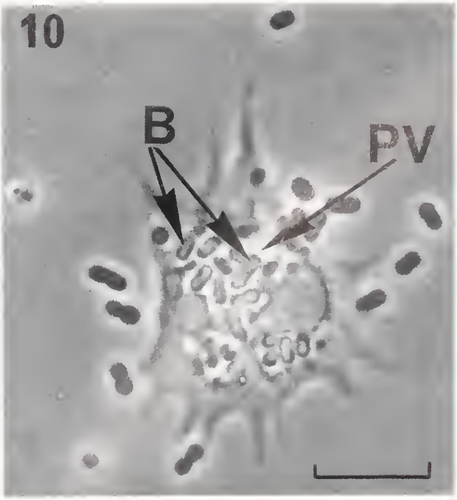
FIGURE 8. Lysed hyaline hemocytes of *A. astacus* after 1 h incubation in 0.1% laminarin. Note the fragmented cytoplasm (cy). Nucleus (N). Phase contrast. Scale bar 10 μm.

FIGURE 9. Hyaline hemocytes of *C. maenas,* after 1 h incubation in 0.1% laminarin. There is some slight vacuolation in the cytoplasm in the perinuclear region (arrows). Phase contrast. Scale bar 10 μm.



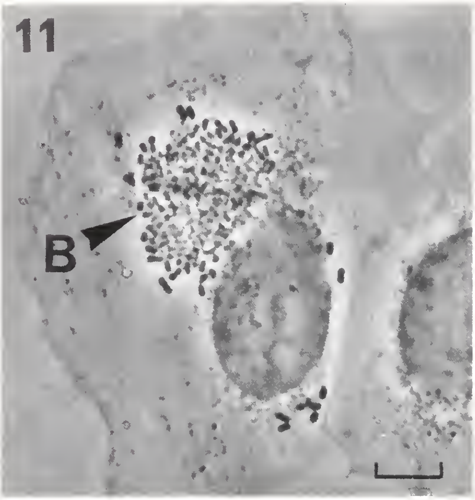


FIGURE 10. Hyaline hemocyte of *C. maenas* incubated with *Moraxella* sp. for 2 h in the presence of 0.1% laminarin. Many intracellular bacteria (B) are enclosed within large phagocytic vacuoles (PV). Phase contrast. Scale bar 10 μm.

FIGURE 11. Hyaline hemocyte of *A. astacus* incubated with 0.1% laminarin. Note the large aggregate of adherent, extracellular *Moraxella* sp. (B) associated with the cytoplasm. Phase contrast. Scale bar 10 μm.

the number of bacteria taken up at 267 (EI = 116) (Table I). The equivalent EI values for *C. maenas* were 118 for the percentage phagocytosis and 101 for the bacteria taken up (Table II).

Dextran, cellulose and chitin also failed to stimulate uptake in *A. astacus*, and in these cultures, the percentage phagocytosis was 5.6% (EI = 101) for dextran, 8.7% (EI = 165) for cellulose and 5.2% (EI = 85) for chitin, compared to 5.3% in the saline incubated controls (Table I). The corresponding values for the number of bacteria ingested were 226 (EI = 112) for dextran, 230 (EI = 114) for cellulose, 207 (EI = 97) for chitin and 201 for the controls (Table I). Cellulose and chitin were particularly unsuitable for studies of this kind because of their insolubility in the culture saline.

Regarding the dose response, treatment of *A. astacus* monolayers with 0.01% laminarin yielded an increase in the percentage uptake of 315 from 3.7% to 12.1% which was similar to the rate of phagocytosis recorded for crayfish with 0.1% laminarin ($P = 0.42$) (Table I). The number of bacteria ingested/100 cells was also increased, from 246 to 446 (EI = 181) which again resembled the response observed with 0.1% laminarin ($P = 0.47$) (Table I). In *C. maenas*, while addition of 0.001% laminarin still raised the percentage phagocytosis significantly above the level obtained for saline incubated cultures ($P = 0.008$), the degree of enhancement was only 202, a value significantly less than that achieved with 0.1% laminarin ($P = 0.002$) (Table II). The number of bacteria taken up by the hemocytes remained at 242 (EI = 112) which did not differ significantly from the number ingested in the saline incubated controls ($P = 0.097$) (Table II).

Concomitant with the enhancement of uptake of *Moraxella* sp. in crayfish and crab hemocytes treated with laminarin, was an increase in the proportion of cells associated with aggregates of ten or more extracellular bacteria (Fig. 11). Although not quantified for every treatment, in *A. astacus* monolayers the percentage asso-

TABLE II

In vitro *phagocytosis of* Moraxella *sp. by hemocytes of* C. maenas

Treatment of monolayers	Percentage phagocytosis		EI[a]	Number of bacteria/100 hemocytes		EI
0.1% laminarin	28.4[b] ± 15.4	$P = 0.008$	303	545 ± 183	$P = 0.008$	200
control[c]	10.0 ± 6.6			279 ± 43		
n = 7[d]						
0.001% laminarin	8.9 ± 4.5	$P = 0.008$	202	242 ± 83	$P = 0.097$	112
control	4.4 ± 2.2			214 ± 56		
n = 7						
0.1% glucose	6.4 ± 2.7	$P > 0.125$	118	220 ± 41	$P > 0.125$	101
control	5.4 ± 2.0			218 ± 74		
n = 10						
preincubation[e] in 0.1% laminarin	4.3 ± 2.1	$p > 0.062$	82	282 ± 91	$P > 0.062$	89
control	5.3 ± 3.3			320 ± 100		
n = 5						

[a] EI as given in Materials and Methods.

[b] Values given are means ± standard deviation.

[c] Controls were incubated in CS.

[d] n = number of animals used in experiment.

[e] Preincubated for 1 h at 20°C before challenge with bacteria in CS.

ciation, as defined by Smith and Ratcliffe (1978), following treatment with 0.1% or 0.01% laminarin, was 40% against *ca.* 3% in the controls. Similar differences were seen in *C. maenas* to 0.1% laminarin, and to a lesser extent with 0.001% laminarin.

It is unlikely that the glucans, alone, were responsible for the agglutination of the test particles, since *Moraxella* sp. suspensions (2.0×10^7 ml^{-1}) incubated for 2 h at 20°C in 0.1% laminarin, glucose or dextran, in the absence of crayfish or crab hemocytes, did not shown any signs of particle aggregation.

Pre-incubation of the hemocytes in 0.1% laminarin prior to bacterial challenge slightly depressed the rate of uptake in *A. astacus* and *C. maenas* (Tables I, II), and in crayfish, the percentage phagocytosis decreased from 2.6% to 2.3%, and EI of 85, with a reduction in the number of bacteria ingested from 235 to 223, an EI of 94 (Table I). In *C. maenas*, the EI values were 82 for the percentage phagocytosis and 89 for the bacteria/100 hemocytes (Table II).

Prophenoloxidase and protease activity in C. maenas *HLS*

Table III shows that *C. maenas* contains prophenoloxidase and protease in the hemocytes. Using L-dopa a substrate, phenoloxidase activity was found to be dependent on Mg^{2+} ions, and inclusion of 50 m*M* $MgCl_2$ with 5 m*M* $CaCl_2$ in the buffers provided the optimal ionic conditions for the reaction to take place (Table III). Pre-incubation of the hemocyte lysate in laminarin (250 μg ml^{-1} final concentration) enhanced the rate of enzyme activity *ca.* fourfold, with similar, but weaker, activation achieved with zymosan (Table III).

Hydrolysing activity against the synthetic chromogenic peptide, Ba-Ile-Glu(γ-O-Piperidyl)-Gly-Arg-PNA, was also slightly improved with Mg^{2+} ions, and addition of β-1,3 glucans to the experimental mixtures enhanced the activity of the enzyme *ca.* threefold.

TABLE III

Phenoloxidase and protease activities in C. maenas *hemocytes*

	Phenoloxidase activity		Protease activity	
	Units/ml	Units/mg protein	ΔA405/30 min	ΔA405/mg protein
HLS in CS (control)	260	5000	0.03	0.6
HLS in 50 m*M* MgCl$_2$[a]	280	5400	0.05	0.9
HLS in CS + laminarin[b]	840	16,200	0.11	2.1
HLS in 50 m*M* MgCl$_2$[a] + laminarin[c]	1040	20,000	0.10	1.9
HLS in 50 m*M* MgCl$_2$[a] + Zs	500	9600	0.08	1.5

[a] = dissolved in distilled water.

[b] = dissolved in CS (250 μg ml^{-1} final concentration during activation).

[c] = dissolved in distilled water (250 μg ml^{-1} final concentration during activation).

Phenoloxidase and protease activity assayed as described in Materials and Methods using L-dopa or the synthetic chromogenic peptide, Bz-Ile-Glu-(γ-O-Piperidyl)-Gly-Arg-PNA as substrate.

C. maenas hemocytes, therefore, appear to possess at least two of the constituent enzymes of the prophenoloxidase activating system, previously described for *A. astacus* (Söderhäll, 1981; Söderhäll, 1982, 1983).

Hemagglutination titres

Weak hemagglutinating activity was found in *C. maenas* serum, but not HLS, for sheep (titre 16–32), horse (titre 4–8) and human A (titre 32) erythrocytes. A lytic factor (titre 8) was present in both serum and HLS for mouse RBC, but neither the hemagglutinins nor the lytic factors were changed by glucan incubation.

Effect of β-1,3 glucans on A. astacus *and* C. maenas *hemocytes* in vivo

The number of circulating hemocytes (THC) in the hemolymph of untreated crayfish was found to be 4.1 × 10^5 ml^{-1} (Fig. 12). Injection of saline significantly raised the THC to 10.3 × 10^5 ml^{-1} at 30 min ($P = 0.001$) and to 9.2 × 10^5 ml^{-1} at 3 h ($P = 0.001$), but by 24 h, the count had returned to 6.9 × 10^5 ml^{-1}, which was not significantly different from untreated animals ($P = 0.016$) (Fig. 12).

Injection of laminarin, however, significantly reduced the THC to 1.7 × 10^5 ml^{-1} within 30 min compared to both untreated ($P = 0.005$) and saline injected controls ($P < 0.001$) (Fig. 12). The THC remained as low as 2.8 × 10^5 ml^{-1} for 3 h ($P = 0.064$ compared to untreated crayfish; $P < 0.001$ compared to saline injected animals) (Fig. 12), but by 24 h had recovered to 3.4 × 10^5 ml^{-1}, which was not significantly different from untreated ($P = 0.38$) or saline treated *A. astacus* ($P = 0.38$) (Fig. 12).

Similarly, in *C. maenas,* injection of saline was followed by an increase in the THC from 26.5 × 10^6 ml^{-1}, the cell count for untreated animals, to 45.7 × 10^6 ml^{-1} at 30 min ($P = 0.05$). After this time, the THC gradually declined to 35.0 × 10^6 ml^{-1} at 3 h ($P > 0.05$) and to 24.0 × 10^6 ml^{-1} at 24 h ($P > 0.05$) (Fig. 13). Inoculation with laminarin produced a *ca.* threefold reduction in the THC to 9.3 × 10^6 ml^{-1} within 30 min ($P < 0.001$ compared to untreated crabs; $P < 0.001$ compared to saline injected controls) (Fig. 13). This decline continued for 3 h, when the THC fell to 3.4 × 10^6 ml^{-1} ($P < 0.001$ compared to untreated *C. maenas;* $P < 0.001$ compared to saline injected controls), but by 24 h, the THC had recovered to 31.3

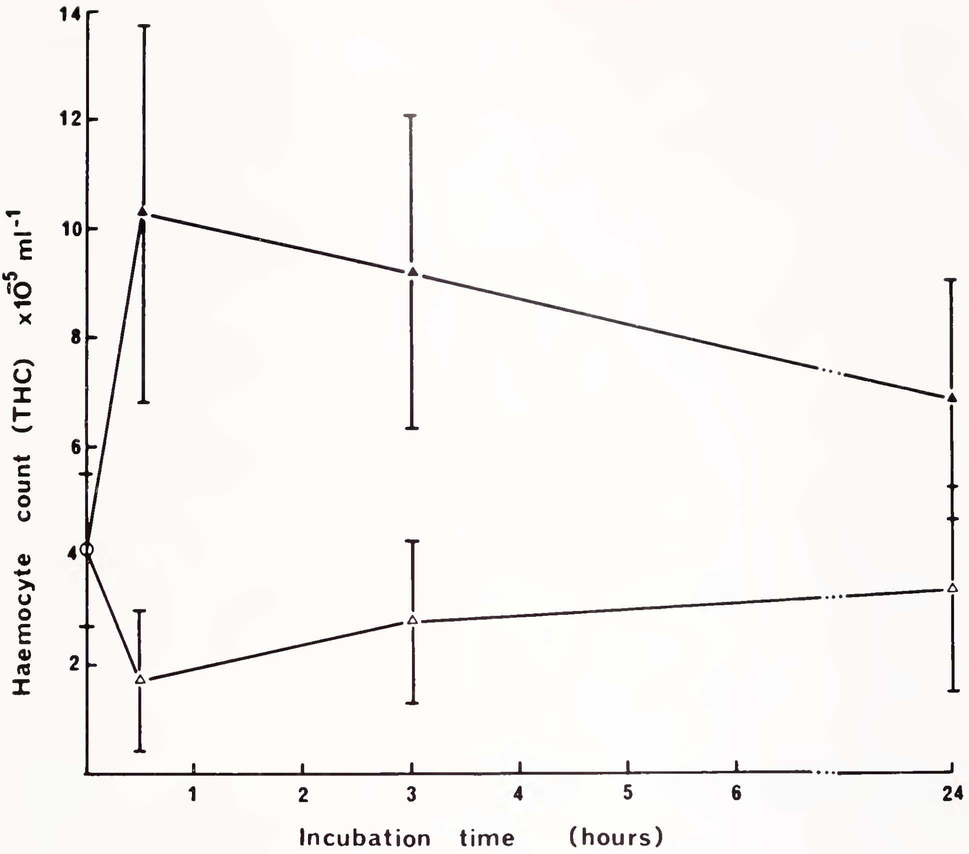


FIGURE 12. Hemocyte count (THC) of *A. astacus* injected with sterile CFS (– ▲ –) or 0.2 mg laminarin/ml hemolymph (– △ –). Injection and bleeding procedure as in Materials and Methods. At each time interval, the values given are means of groups of 8 animals ± standard deviation. The time zero count (– ○ –) was obtained from 8 untreated animals.

$\times 10^6$ ml^{-1}, which was not significantly different from untreated ($P > 0.05$) or saline injected crabs ($P = 0.03$) (Fig. 13).

Glucan injection was rarely fatal for the crayfish or crabs, but glucan inoculated animals were often lethargic, and the hemolymph samples from the experimental animals tended to clot more rapidly than those from untreated or saline injected controls.

DISCUSSION

The results presented in this paper show that β-1,3 glucans profoundly affect the behaviour of crayfish and crab hemocytes by enhancing the rate of phagocytosis of bacteria *in vitro*, and causing a marked reduction in the number of circulating cells *in vivo*. Since the responses observed *in vitro* were elicited only by laminarin, and not by glucose or other glycans, the elevated rates of uptake exhibited by the cells could not have been due simply to the nutritive properties of the β-1,3 glucans, and, as the *in vivo* reaction was similar to that seen previously in *C. maenas* following injection of bacteria (Smith and Ratcliffe, 1980a) a cellular defense reaction appears

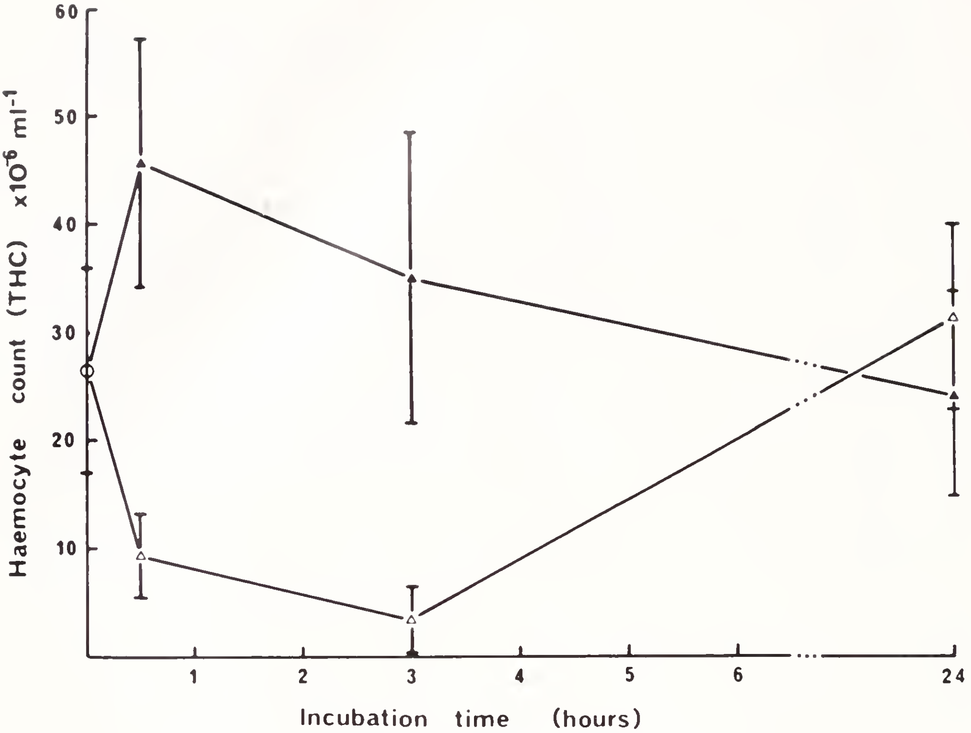


FIGURE 13. Hemocyte count (THC) of *C. maenas* injected with sterile CS (– ▲ –). Injection and bleeding procedures as in Materials and Methods, and at each time interval, the values given are means of groups of 8 animals ± standard deviation. The time zero count (– ○ –) was obtained from 10 untreated crabs.

to have been initiated. In mammals, β-1,3 glucans are known to promote a hyperphagocytic state *in vivo* (Di Luzio, 1979) and to activate macrophages directly *in vitro* (Seljelid *et al.*, 1981), but this is the first report of their effect on cell behavior in invertebrates.

Regarding the mechanism(s) through which the glucans exert their influence in crustaceans, it is unlikely that they act through specific agglutinin mediated receptors on the hemocyte surface as proposed for opsonic phenomena reported in other vertebrates (McKay and Jenkin, 1970; Renwrantz and Cheng, 1977), since agglutinating activity against *Moraxella* sp. is absent from *C. maenas* serum (Smith and Ratcliffe, 1978), and, in the present study, changes in the titre of erythrocyte agglutinins were not detected following glucan incubation. Instead, in *A. astacus*, β-1,3 glucans are known to specifically activate prophenoloxidase, in the hemocytes, through a complex enzyme cascade involving at least one serine protease (Söderhäll, 1982, 1983) and a similar system, also activated by β-1,3 glucans, appears to exist in *C. maenas*. After activation, phenoloxidase is sticky and will attach to many different types of foreign surface including glass, plastic or fungal spores (Söderhäll *et al.*, 1979), so that it could have facilitated the binding and subsequent ingestion of the bacteria by the cells in the present study. Certainly, in *A. astacus* the glucans were seen to promote degranulation of the cells in the monolayers, and although it was impossible to observe equivalent events in *C. maenas* hemocytes, addition of L-dopa to the crab monolayers following glucan treatment confirmed the presence of phenoloxidase on the coverslip surface. Further support for the opsonic role of the hemocyte proteins has also recently been provided by Söderhäll, Vey, and Ramstedt (submitted for publication) who showed that crayfish haemocytes mount stronger encapsulation responses *in vivo* to fungal spores coated with hemocyte lysate supernatant than to fungal spores coated in plasma or buffer. Also in *C. maenas*, electron-dense material, identified as melanin, has been noted around *Moraxella* sp. enclosed within hemocyte clumps 24 h after injection of the bacteria into the hemocoel (Smith and Ratcliffe, 1980b, and unpubl.)

Curiously, when the hemocytes of *A. astacus* and *C. maenas* were preincubated with β-1,3 glucans, the opsonizing capacity of the prophenoloxidase activating system was lost, probably because the sticky proteins had already attached to the glass surface of the coverslips and were unavailable to the bacteria.

In arthropods, phenoloxidase activity, as evidenced by melanin deposition, frequently accompanies the host cellular reactions, to wounding, parasites, foreign implants or injected test particles (Salt, 1970; Ratcliffe and Rowley, 1979; Vey, 1979), and Söderhäll (1982) has proposed that the activation process may constitute an important recognition system in these animals. It would therefore be interesting to determine whether β-1,3 glucans also have a potentiating influence on the hemocytic responses of other arthropod species, such as the silk moth, *Bombyx mori*, which possesses prophenoloxidase that can be activated by β-1,3 glucans (Ashida, 1981). Moreover, β-1,3 glucans represent just one class of non-self molecules present in the cell walls of fungi (Unestam and Söderhäll, 1977), and prophenoloxidase can be triggered by other foreign molecules, for example bacterial lipopolysaccharide (endotoxin) (Söderhäll and Häll, unpublished) which causes gelation of amoebocyte lysates of the horseshoe crab *Limulus polyphemus* (Levin and Bang, 1964).

Finally, in mammals, β-1,3 glucans are known to activate complement through the alternative pathway (Hamuro *et al.*, 1978; Reid and Porter, 1981), and Seljelid *et al.* (1981) have suggested that the increased cellular metabolism; measured by increased glucosamine incorporation, decreased nucleotidase activity and cell enlargement; induced in macrophages *in vitro* by treatment with glucans could be achieved through complement intervention. At present, direct-acting hemolytic complement has not been identified in any invertebrate group (Anderson, 1981), but Day *et al.* (1970) have predicted that some might possess the terminal components which make up the alternative pathway. In arthropods, the prophenoloxidase system, in comprising a complex enzyme cascade activated by β-1,3 glucans (Unestam and Söderhäll, 1977; Söderhäll, 1981, 1982, 1983), initiating coagulation (Söderhäll, 1981), possessing antimicrobial properties (Söderhäll and Ajaxon, 1982), and, as indicated in this study, functioning as an opsonin, shows many similarities to complement and could represent such a primitive form. Related systems may have also evolved in other invertebrates, but their description awaits further investigation.

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SIZE- AND DIET-RELATED VARIATIONS IN ENZYMIC ACTIVITY AND TISSUE COMPOSITION IN THE SABLEFISH, *ANOPLOPOMA FIMBRIA*

KATHLEEN M. SULLIVAN* AND GEORGE N. SOMERO

Marine Biology Research Division, A-002, Scripps Institution of Oceanography,
University of California, San Diego, La Jolla, CA 92093

ABSTRACT

The influences of body size, ration size, season, and latitude on several biochemical properties of skeletal muscle and liver were examined in the sablefish, *Anoplopoma fimbria,* to understand how these factors interact to establish the biochemical and physiological condition of the organism. Activities in muscle of the glycolytic enzymes lactate dehydrogenase (LDH) and pyruvate kinase (PK) increased significantly with increasing body size. This size-dependent variation in enzymic activity must be corrected for when the effects of other enzyme activity influencing variables, *e.g.*, ration size, are studied. Ration size had a strong influence on muscle enzyme activities and on the water and lipid content of muscle. Starvation led to large decreases in glycolytic enzyme activity and lipid content. The LDH content of muscle was directly proportional to the amount of food ingested (expressed as percent body weight ingested per day). Latitudinal differences among sablefish collected between Alaska (Bering Sea) and southern California and seasonal differences among individuals collected off southern California also may be due to varying ration quantity and quality. During starvation, lipid reserves in muscle appear to be mobilized as a major energy source in preference to protein.

INTRODUCTION

Recent studies of marine teleost fishes have shown that certain biochemical characteristics of white skeletal muscle, notably enzymic activity (expressed as units per gram wet weight) and protein content, correlate strongly with important ecological and physiological attributes of the species. Comparisons of species having different depths of occurrence have shown that the activities of two glycolytic enzymes (lactate dehydrogenase [LDH] and pyruvate kinase [PK]) decrease by two to three orders of magnitude with increasing depth of occurrence (Childress and Somero, 1979, Sullivan and Somero, 1980; Siebenaller and Somero, 1982; Siebenaller *et al.*, 1982). Because LDH and PK activities reflect the capacity of a fish for vigorous high speed swimming (Somero and Childress, 1980; Sullivan and Somero, 1980), these depth-related changes in glycolytic activity are interpreted to reflect greatly reduced locomotory capacities in deeper-living fishes. Several differences in LDH and PK activity were also found among fishes living at the same depths, and these differences can be interpreted in terms of different locomotory and feeding habits among the species. Another major contributor to the level of glycolytic activity in fish muscle is body size. Somero and Childress (1980) showed that the activities

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* Present address: School of Natural Resources, Samuel Trask Dana Building, University of Michigan, Ann Arbor, MI 48109, and Laboratory of Limnology, University of Wisconsin, Madison, WI 53706.

of LDH and PK increased significantly with increasing body size within a species, while enzymes of aerobic metabolism (citrate synthase [CS] and malate dehydrogenase [MDH]) typically decreased in larger individuals of a species. The increased glycolytic capacity per gram muscle in larger individuals of a species is regarded as a mechanism for maintaining a constant capacity of burst swimming, measured in body lengths swum per unit time, in all sizes of fish of a species. Two additional contributors to muscle enzyme activities are condition and dietary influences. For example, muscle LDH activities in field caught anchovies (*Engraulis mordax*) were sevenfold higher than activities in muscle of laboratory reared anchovies, a difference which may derive from conditioning (Somero and Childress, 1980). Conditioning effects on fish muscle enzymic activities have also been reported by Johnston and Moon (1980). Dietary influences on muscle enzyme levels may also be significant. Patterson *et al.*, (1974), Jobling (1980) and Moon and Johnston (1980) have shown that starvation leads to alterations in enzymic activity and, in some instances, in levels of the contractile apparatus.

The present study was undertaken to examine the effects of several of the variables known to influence muscle enzymic activities, namely body size, diet and depth of occurrence, on a single teleost species, the sablefish (*Anoplopoma fimbria*). Through study of field specimens and of fish held under controlled conditions in the laboratory we hoped to learn more about the degree of variation in muscle biochemistry that can occur within a species. This information could be useful for developing biochemical indices for gauging the physiological state of field caught sablefish, and these data also might be of benefit in establishing the relative importance of different factors, *e.g.*, body size *versus* diet, in establishing muscle biochemical properties. The variation in biochemical properties observed within a single species also would shed light on the extent to which interspecific comparisons could be valid.

Anoplopoma fimbria was selected for this study because of its wide latitudinal and depth distributions, its capacity to survive under widely varying dietary conditions (Sullivan and Smith, 1982), and its suitability for long-term aquarium holding. The sablefish is a benthopelagic species which occurs on the continental slope from the Bering Sea, Alaska, to off Cedros Island, Baja California, Mexico. It occurs in shallow water (down to 50 m depth) at the northern end of its range, and in relatively deep water at the southern end of its range (down to approximately 1500 m off San Diego, California). There is extensive information available in the literature about the life history and food habits of this species throughout its range (Conway, 1967; Phillips, 1969).

For our study, specimens of *A. fimbria* were collected over much of the species' distribution limits: the Bering Sea, off the Queen Charlotte Islands (British Columbia), Monterey Bay (California), and in the Southern California Bight. All of these areas were sampled at the same time of the year (June–July), and specimens also were collected throughout the year off southern California. A wide size range of fish was obtained to investigate size-specific ("scaling") effects on muscle biochemistry. Aquarium studies were conducted with southern California specimens to examine the effect of ration on muscle biochemistry.

Our results indicate that the biochemical properties (enzymic activity, and protein, lipid, and water contents) of both white skeletal muscle and, to a lesser extent, liver are strongly affected by ration. Seasonal and latitudinal variations in muscle biochemistry seem explainable largely in terms of food availability. Glycolytic enzyme activity in white skeletal muscle is size-dependent. Thus, size-related changes must be segregated in studies of dietary effects.

Materials and Methods

Sablefish were collected from four sites along the west coast of North America: Bering Sea, Alaska (58°N, 50 m), Queen Charlotte Islands (52°40′N, 90–210 m), Monterey Bay, California (37°30′N, 405 m) and the Southern California Bight Area (two stations: Tanner Basin 32°43′N, 1200 m and San Diego Trough 32°53′N, 1060 m). Fish were collected by trap line, free vehicle set lines (FVSL), and trawls. All fish were measured and weighed, and then muscle and liver tissues were removed and frozen (dry ice) for laboratory analysis. Fish for laboratory holding were collected off San Diego, California at a depth of 468 m, and held as described in Sullivan and Smith (1982). Fish in the laboratory were fed weekly on a diet of chopped squid and mackerel, with records kept of grams ingested by each fish. Five fish were starved, six fish were fed 7% of their wet body weight per week, and six fish were fed 15–18% of their wet body weight per week. After twenty-four weeks in the laboratory, the fish were sacrificed and assayed by the same procedures used for field fish. All muscle samples were taken from a site in the epaxial white musculature behind the gill cover and well above the lateral line. Skin and any red muscle tissue were removed.

Water contents were determined from two 1-gram muscle samples taken from frozen tissue according to the procedure of Sullivan and Somero (1980). Protein concentration of the white muscle was determined using a modified microburet method (Itzhaki and Gill, 1964). Bovine serum albumin was used as a standard. Lipid content of the tissue was determined by a chloroform/methanol extraction and a simple charring method (Marsh and Weinstein, 1966). Tripalmitin was used as a standard. All values are expressed as percent of tissue wet weight.

For the assays of enzymic activity, tissue samples were dissected while frozen, weighed, added immediately to 8 volumes of homogenization buffer (10 m*M* Tris-HCl, pH 7.5 at 10.0°C) at ice bath temperatures (0–4°C), and homogenized in conical ground glass-surfaced homogenizers ("Duall type 23"; Kontes Glass Co., Vineland, New Jersey, USA) driven by hand. The homogenates were centrifuged at 2500 × *g* for 15 minutes and the supernatant fractions pipetted into test tubes, avoiding any superficial lipid layer, and used without additional purification. Further dilution of the homogenates was not necessary.

Activities of LDH and PK in white muscle, and MDH and CS in the liver were measured at 10.0°C (±0.2°C) using freshly prepared homogenates according to the methods of Somero and Childress (1980). Substrate and cofactor concentrations were adjusted to give optimal reaction velocities. All enzyme activities are expressed in international units (μmoles substrate converted to product per minute) per gram of wet (or when noted, dry) weight at 10.0°C. Most discussion of enzyme activities refers to values based on activity per gram of wet weight. This seems most appropriate for estimating the metabolic potential of the tissue. However, activities per gram dry weight are used to factor out the variability in water content of the muscle tissue when examining the scaling effect of glycolytic enzymes with increased size.

Results

Because the specimens used in this study ranged in weight from 430 g to 3600 g, and in forklength from approximately 40 cm to 80 cm (see Fig. 1), we examined possible size-related ("scaling") relationships between body size and glycolytic enzyme activity. To this end we plotted LDH (Fig. 1) and PK (Fig. 2) activities as a function of forklength for all southern California specimens, the largest group of sablefish used in our studies.

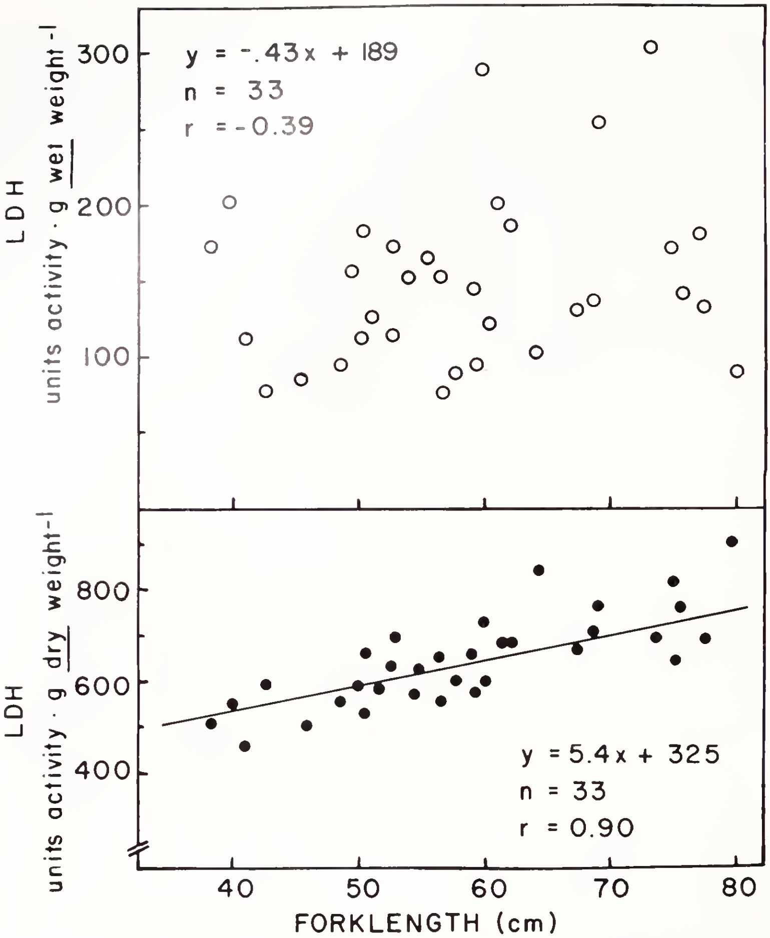

FIGURE 1. A) Lactate dehydrogenase (LDH) activity per gram wet weight in white skeletal muscle of *Anoplopoma fimbria* collected off southern California *versus* forklength. The equation generated by a linear regression is given, with the regression coefficient. B) LDH activity per gram dry weight for the same *Anoplopoma fimbria versus* forklength. The equation generated by a linear regression is given.

Forklength was used rather than body mass because of the greater accuracy in measuring length at sea. Fish caught by FVSLs often retained large quantities of water in their guts, making weight measurements even less reliable. LDH activity *versus* forklength for 33 fish collected off southern California is plotted in units of activity per gram wet weight and per gram dry weight (Fig. 1). The data encompass the full range of sizes studied and include data on individuals collected at different times of the year. LDH activity per gram wet weight does not correlate strongly with forklength ($r = -0.39$). However, converting all the LDH activities to units of activity per gram dry weight yields a strong positive correlation between enzyme activity and forklength ($y = 5.4x + 325$; $r = 0.90$). Similarly, when expressed in terms of units of activity per gram dry weight, PK activity also scales strongly with body size (Fig. 2). The scatter noted in enzymic activities expressed in terms of gram wet weight of tissue is largely a reflection of variation in water content among specimens; water content of white muscle ranged from 62% to 91%, but showed no systematic

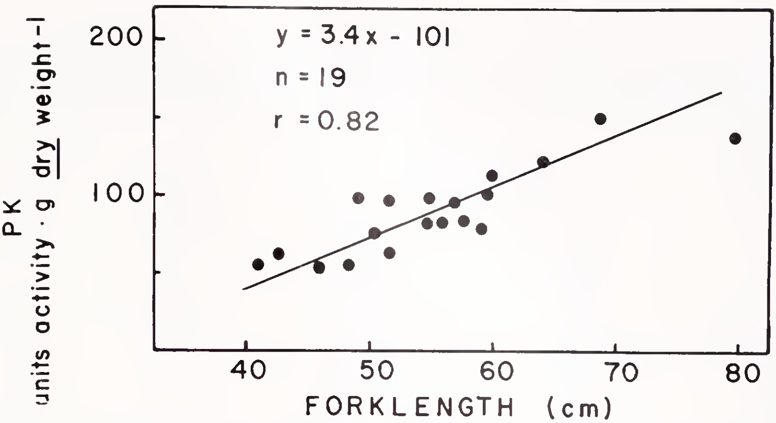

FIGURE 2. Pyruvate kinase (PK) activity (units per gram dry weight) *versus* forklength for *Anoplopoma fimbria* collected off southern California. The equation generated by a linear regression is given, with the regression coefficient.

variation with body size (multiple regression analysis). Thus, while differences among individuals in muscle water content contribute to the variation in enzymic activity (expressed per gram wet weight), the only significant trend noted in this analysis is between length and enzymic activity expressed on a dry weight basis. The fact that significant correlations between enzymic activity on a wet weight basis were not found in the sablefish but were found in most other fishes studied may be due to the unusually large variation in water content found in the sablefish relative to other fishes so examined (*cf.* Siebenaller and Somero, 1982; Siebenaller *et al.*, 1982).

For all comparisons of LDH and PK activities in different experimental groups we performed a correction for size-dependence of enzymic activities on the measured enzymic activities. For example, if a sablefish 66 cm in forklength was found to have a muscle LDH activity of 130 units activity per gram wet weight, and to have 82% water in its white muscle, the units of activity per gram dry weight would be 722 (130 × 100/18 = 722). From the linear regression, the LDH activity increases by 5.4 units per gram dry weight for each centimeter increase in length. All fish have been standardized by convention to a 55 cm "reference fish." Thus, correcting the LDH activity of this 66 cm fish to 55 cm involves subtracting 6 units of LDH activity (5.4 units/cm × 11 cm = 6 units), to yield a value of 716 units per gram dry weight. Converting back to units per gram wet weight, the size corrected LDH activity is 128. All LDH and PK activity measurements were converted back to units per gram wet weight for comparison to values in the literature.

Although the size correction is not large for specimens near the average length (128 compared to original value of 130 units LDH activity per gram wet weight in the example chosen), this correction may be important in comparing fish at two extremes of the size range examined.

Table I gives the muscle water, protein and lipid content, and muscle LDH and PK activities for fish collected from the four locations on the west coast of North America. All fish were collected during the months of June and July, thus removing variation due to seasonal effects. The fish were collected at successively deeper stations in moving from north to south (see Methods). Factors influencing muscle and liver biochemistry could include not only depth *per se*, but also latitudinal differences in food abundance or available prey species. Fish from the three northern stations

TABLE I

Average muscle water, protein and lipid content, and muscle lactate dehydrogenase (LDH) and pyruvate kinase (PK) activities per gram wet weight (with standard deviations) for Anoplopoma fimbria *collected at four stations along the west coast of North America*

Station (sample size)	Muscle composition[a]		LDH[b]	PK[b]
Bering Sea (n = 16)	Water	77.7 ± 2.7%	166 ± 59	24.2 ± 10.2
	Protein	15.0 ± 2.8%		
	Lipid	5.1 ± 3.2%		
Queen Charlotte Islands (n = 27)	Water	70.7 ± 5.5%	181 ± 46	17.2 ± 6.0
	Protein	17.0 ± 6.6%		
	Lipid	9.3 ± 2.7%		
Monterey Bay (n = 8)	Water	77.8 ± 4.0%	134 ± 67	17.4 ± 9.0
	Protein	15.1 ± 2.0%		
	Lipid	3.0 ± 2.0%		
San Diego, CA (n = 26)	Water	79.0 ± 6.0%	112 ± 64	15.7 ± 10.0
	Protein	11.3 ± 4.0%		
	Lipid	6.4 ± 3.0%		

[a] Muscle composition is in percent wet weight of tissue.
[b] International units per gram wet weight of tissue, and size corrected (see text).

have significantly lower muscle water content, and higher protein content (one-way ANOVA, $\alpha = 0.05$). The three northern populations also have a significantly higher muscle LDH activity, but there is no significant difference in muscle PK values (one-way ANOVA, $\alpha = 0.05$). The highest LDH activities were in the fish collected off the Queen Charlotte Islands, and there is a decrease in the average LDH activity moving south from this station.

The composition of liver tissue taken from fish collected in June and July in the Bering Sea, off the Queen Charlotte Islands and off San Diego, California, is given in Table II. There is no significant difference in the water content of the liver tissue among the three stations (one-way ANOVA, $\alpha = 0.05$); however, for the fish collected off San Diego the protein content was significantly higher and the lipid content was significantly lower than for fish from the two northern stations. Lipid values were highest in the fish collected off the Queen Charlotte Islands. The variability in water content of the liver tissue was much lower than that of the white muscle tissue.

TABLE II

Proximate analysis of liver tissue from Anoplopoma fimbria *collected in the Bering Sea, off the Queen Charlotte Islands, British Columbia and off San Diego, California*

Station (sample size)	Percent water	Percent protein	Percent lipid
Bering Sea (n = 10)	63.9 ± 4.5	16.3 ± 3.0	15.5 ± 2.6
Queen Charlotte Is. (n = 10)	55.0 ± 4.5	21.6 ± 5.6	18.1 ± 4.1
San Diego, CA (n = 17)	59.3 ± 5.4	23.1 ± 3.5	10.4 ± 2.9

Percent wet weight of water, lipid and protein is given with the standard deviation. All fish were collected in the summer months of June and July.

TABLE III

Seasonal changes in white skeletal muscle composition and glycolytic enzyme activities for Anoplopoma fimbria *collected off San Diego, California*

Month (sample size)	Muscle composition[a]		LDH[b]	PK[b]
February (n = 14)	Water	78.0 ± 2.4	102 ± 31	11.3 ± 3.6
	Protein	13.0 ± 3.1		
	Lipid	5.0 ± 1.5		
August (n = 12)	Water	71.2 ± 1.8	158 ± 64	17.6 ± 5.7
	Protein	15.0 ± 1.0		
	Lipid	12.0 ± 3.2		
December (n = 16)	Water	76.1 ± 3.6	149 ± 65	16.2 ± 5.3
	Protein	11.0 ± 3.2		
	Lipid	10.0 ± 3.1		

Fish collected in the months of February, August and December were assayed separately.

[a] Average percent wet weight of water, protein and lipid in the white muscle is given with standard deviation.

[b] International units activity per gram wet weight (corrected for size).

After making size and latitudinal comparisons of muscle composition and enzyme activities, fish collected at the San Diego site throughout the year were examined for seasonal changes in muscle biochemistry. Table III lists the white muscle water, protein and lipid contents, and muscle LDH and PK activities per gram wet weight, size corrected, for fish collected in February, August, and December. The water content in August fish is significantly lower than in fish collected in December and February (one-way ANOVA, $\alpha = 0.05$) and the lipid content is significantly lower in February fish. The white muscle LDH activity is also significantly lower in the fish caught in February; there is no difference in the muscle LDH levels between fish caught in August and in December. There is no significant difference in muscle PK activities among fish caught at different times, but the average PK activity value is lowest for fish caught in February.

To assess the role of diet in both the seasonal and geographic variation of muscle composition and glycolytic enzyme activities of field-caught fish, 17 sablefish were held in the laboratory for 24 weeks to examine dietary-induced changes in muscle, liver and gonad composition, and muscle and liver enzyme activities. Table IV presents the results of this experiment. Three dietary treatment groups, starved, half-ration and full-ration, were used. Enzyme activities and tissue composition values were averaged within groups. Starved fish had significantly higher water content, and lower lipid and protein content in muscle than half- and full-ration fish (one-way ANOVA, $\alpha = 0.05$). There was no statistical difference in any parameter for full and half-ration fish (Student's t, $P = 0.05$). Starved fish have significantly lower muscle LDH and PK activities, but do not differ significantly from fed fish in liver MDH and CS activities or liver protein content.

Although there were no differences in the biochemical parameters of the muscle, liver and gonad of full and half-ration fish, half-ration fish lost weight during the experimental period, while the group of fish on a full-ration gained an average of almost 300 g. Growth rates in laboratory full-ration fish were 25% higher than known field growth rates (Sullivan and Smith, 1982). Starved fish not only lost weight, but suffered loss of muscle protein and lipid, and had higher water contents in liver and muscle tissues. There was no difference in the composition of gonad tissue between

TABLE IV

Enzyme activities and tissue composition for Anoplopoma fimbria *kept in the laboratory for 24 weeks on variable rations*

	Starved n = 5	Half n = 6	Full n = 6
Average Ingestion			
Rate (g/wk)	0	59 ± 23	154 ± 45
% Ration[a]	0	5.0	10.2
White Muscle[b]			
Water	81.8 ± 0.9	73.6 ± 1.1	71.6 ± 1.7
Protein	10.0 ± 0.6	12.5 ± 1.7	11.6 ± 1.4
Lipid	2.0 ± 0.8	11.6 ± 1.9	15.2 ± 2.7
Lactate Dehydrogenase[c]	46.2 ± 22	146 ± 36	177 ± 27
Pyruvate Kinase[c]	7.1 ± 4	25 ± 13	27 ± 5
Liver[b]			
Water	78.9 ± 1.9	64.1 ± 7.9	67.6 ± 8.4
Protein	14.2 ± 2.8	19.3 ± 3.1	14.7 ± 3.4
Lipid	3.0 ± 1.6	13.0 ± 4.2	13.4 ± 3.1
Malate Dehydrogenase	133 ± 32	155 ± 55	141 ± 41
Citrate Synthase	2.1 ± 0.5	2.1 ± 0.6	2.0 ± 0.6
Gonad[b]			
Water	85.9 ± 0.8	83.5 ± 3.2	82.5 ± 1.7
Protein	6.8 ± 0.7	5.7 ± 2.6	9.3 ± 1.2
Lipid	1.2 ± 0.6	3.2 ± 1.1	3.9 ± 1.2
Average Initial Weight	1117 grams	1348 grams	1220 grams
Average Final Weight	1000 grams	1197 grams	1505 grams

All values are averaged for each of the three treatment groups.

[a] Fish were fed weekly either a half ration (5–7% of wet body weight) or a full ration (15–17% wet body weight).

[b] Percent wet weight of water, protein, and lipid are given for white skeletal muscle, liver, and gonad with standard deviations.

[c] Muscle glycolytic enzyme activities are size corrected.

the groups. Gonadal tissue made up less than 2% of the total body wet weight in all fish.

The relationship between muscle LDH activity per gram wet weight (size corrected) and ration was further examined by plotting the enzyme activities from individual fish from the laboratory experiment against the average percent ration (percent of wet body weight ingested per week) ingested during the 24-week period (Fig. 3). Though the sample size is small, there is a positive correlation between ration and LDH activity. Both a linear regression and non-linear logarithmic curve were fitted to the data. The logarithmic curve ($y = 58.6 + 106 \log x$) gave the best fit ($r = 0.84$).

DISCUSSION

The relationship between the weight specific oxygen consumption rate and body weight of the sablefish has been determined from laboratory studies (Sullivan and

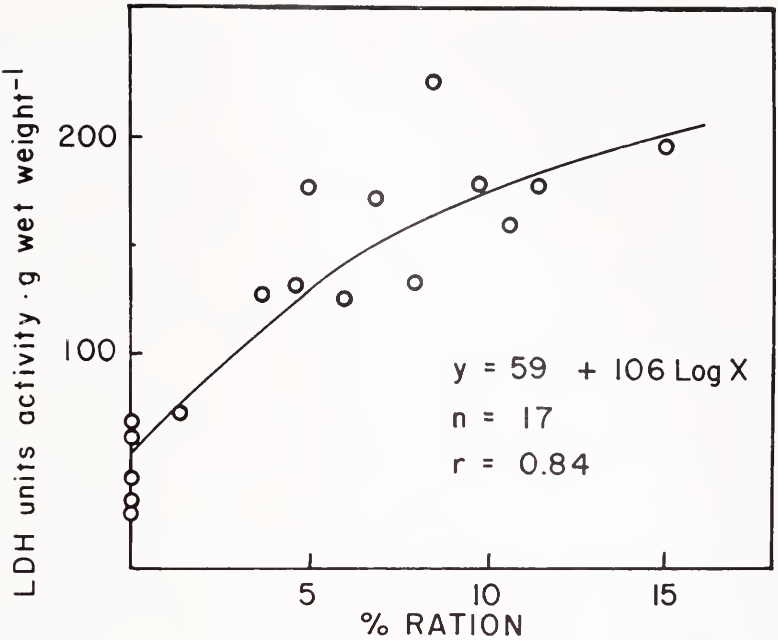

FIGURE 3. Average % ration (percent of wet body weight ingested per week) *versus* white muscle LDH activity per gram wet weight, size corrected for 17 *Anoplopoma fimbria* held in the laboratory for 24 weeks. The logarithmic curve was drawn from the equation y = 58.6 + 106 log x. The regression coefficient (r) is 0.84.

Smith, 1982). The exponent of the equation $\dot{V}_{O_2} = aM^b$ where $\dot{V}_{O_2}$ is the weight specific oxygen consumption, a is a constant, and M is body mass is −0.28. This exponent is not significantly different from −0.26, the scaling coefficient observed for weight specific oxygen consumption for different sized animals (Hemmingsen, 1960; Schmidt-Nielson, 1975). In contrast, the scaling coefficient b for the weight specific LDH activity per gram dry weight of muscle is 0.66. That is, the LDH activity per gram dry weight *versus* forklength data can be fit to a power curve, generating the equation $y = 42x^{0.66}$ where y is LDH activity per gram dry weight and x is forklength. Obviously, the scaling of LDH activity per gram dry weight differs significantly from the scaling of oxygen consumption per unit weight. Somero and Childress (1980) attributed the positive scaling of glycolytic enzymes in other marine teleost species to increased power demands for burst swimming by larger fish of a species. The increase in glycolytic enzyme activity with increasing body size in many of the fishes studied matches closely the predicted increase in swimming power needed to maintain a constant relative burst swimming ability, measured in body lengths swum/unit time over a range of sizes for a given species. Observations of sablefish from submersibles indicate that this species is capable of very fast bursts of swimming when startled by light or disturbances (Sullivan, personal observations). The finding of fast-swimming pelagic organisms, *e.g.*, squid, in sablefish gut contents (Conway, 1967) likewise argues for a robust swimming ability by this fish. Furthermore, the absolute amount of glycolytic activity in sablefish muscle is high, *i.e.*, greater than 100 units LDH activity per gram wet weight relative to other deep-living fishes (*i.e.*, lower than 60 units LDH activity per gram wet weight) examined (Sullivan and Somero, 1980). Thus, selection for a relatively high and size-independent burst swimming ability in sablefish may account for the observed scaling of LDH and PK activities in white skeletal muscle. Because of the strong scaling of LDH and PK activities per gram muscle with body size (Fig. 1), it is important to correct for the effect of body size in studies where another experimental variable, *e.g.*, diet, capable of affecting muscle enzymic activity is being examined. This correction was made in all cases in our study.

Partitioning the variation in LDH and PK activities to other factors than size led us to examine, first, latitudinally separated sablefish populations which have different depths of occurrence. Latitudinal and/or depth effects appear to have some influence on the activities of these enzymes (Table I). For sablefish collected during the summer months, the mean LDH activity per gram wet weight of muscle was 181 for fish caught off the Queen Charlotte Islands. In contrast, the LDH activity per gram wet weight muscle for fish caught off San Diego was 112; this difference was found to be significant (student's t, $\alpha = 0.05$).

Considering the low lipid content of the muscle and liver tissues of southern California fish, and the large change in muscle glycolytic enzyme activities induced by laboratory starvation, it is most likely that differences seen in the latitudinal comparison of muscle biochemistry in sablefish are induced by dietary rather than depth differences.

Dietary differences may also account for the seasonal changes observed in sablefish muscle biochemistry (Table III). For sablefish collected from the same station off southern California at different times of the year, a change in the muscle composition and enzyme activities is seen at times corresponding to changes in prey availability. Information collected by Conway (1967) on the stomach contents of fish collected throughout the year suggests seasonal changes in prey availability and feeding habits. In February, there is low abundance of two prey species, the fish, *Sebastolobus alascanus*, and the squid, *Loligo opalescens;* ophuiroids and salps are found in stomach samples at this time (Conway, 1967). In February, the lowest LDH activities were observed for sablefish white muscle, along with low muscle lipid content. In August, the young of the year of *S. alascanus* are available as prey items, and in December, many squid are spawning in the submarine canyons. Thus, during these two months, there appears to be a higher abundance of two common prey items (Conway, 1967), and the greatest muscle LDH activities are seen for sablefish at this time. These high muscle LDH activities can be viewed as an effect of an increased ration size or feeding frequency and as an adaptation for increasing the fish's swimming capacity, *i.e.*, its ability to obtain the fish and squid captured at this season.

To analyze further the dietary contribution to muscle enzyme activities, sablefish were kept in the laboratory on various ration sizes and sacrificed after 24 weeks. Compositional analysis of liver, muscle and gonad tissue shows that the muscle is the most responsive tissue to various rations (Table IV). The muscle LDH and PK activities of starved fish were on an average 20% and 25%, respectively, of the activities of fed fish. Muscle water content was significantly higher and lipid content was significantly lower in starved *versus* fed fish.

Jobling (1980) found that in starved plaice (*Pleuronectes platessa*) the muscle tissue was first depleted of glycogen and lipid stores, then the liver was seen to decrease in lipid content. Loss of integrity of liver tissue (higher water content, lower protein and lipid content) was frequently followed by death in starved plaice (Jobling, 1980). It was not possible to assay for glycogen in sablefish tissues since many of the field caught fish struggle vigorously on the FVSLs, and fish were not killed quickly enough to preserve tissue metabolite levels. Patterson *et al.* (1974) noted that plaice serially slaughtered during starvation suffer a loss of integrity in muscle tissue first, and that white muscle contractile proteins are preferentially utilized by the fish during starvation. With starvation, liver lipid stores are also depleted in sablefish, but there appears to be no change in liver oxidative enzyme activities between starved and fed sablefish. Thus, the activities of liver enzyme are preserved during dietary changes as well as during depth-related changes. Muscle glycolytic enzyme activities, muscle water content, muscle lipid content and liver lipid content appear to be the most informative parameters reflecting the dietary history of the fish.

On the basis of laboratory experiments, one can speculate on the relative ration sizes of field caught fish. For example, fish collected off the Queen Charlotte Islands have the highest average muscle LDH and PK activities, muscle lipid content and liver lipid content. Their ration levels may be higher than the 15% ration (percent wet body weight eaten per week) fed laboratory fish as judged from the higher enzyme activities and lipid contents of muscle and liver. However, laboratory experiments do not take into account energy expended in swimming activity, since fish in the aquarium were kept in confined areas to minimize activity. In light of the laboratory experiment results, the magnitude of change seen in muscle and liver biochemical parameters between northern and southern populations of sablefish could easily be explained by dietary differences, or depth-related changes in food abundance. There is a very strong positive correlation between muscle LDH activity and ration size (Fig. 3) suggesting that this relationship may follow a predictable trend much like other effects of ration on growth and composition. More experiments are necessary to determine the exact nature of the correlation (logarithmic *versus* linear), but the ration size could possibly be determined from muscle enzyme activity information.

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A QUANTITATIVE ANALYSIS OF THE ANNUAL TESTICULAR CYCLE OF THE BRITTLE-STAR *AMPHIPHOLIS KOCHII* BY MEANS OF AUTORADIOGRAPHIC INVESTIGATION

MASAKANE YAMASHITA AND FUMIO IWATA

Zoological Institute, Faculty of Science, Hokkaido University, Sapporo 060, Japan

ABSTRACT

This paper concerns the annual testicular cycle of the brittle-star *Amphipholis kochii* (Echinodermata: Ophiuroidea). An autoradiographic study was applied for analysis of the seasonal changes in the number of spermatogenic cells, the duration of spermatogenesis and the spermatocyte production rate. The annual testicular cycle can be divided into five phases; spermatogenesis occurs during phases 2 to 4 (November to June). Spermatogenesis during phases 3 and 4 (March to June) was sensitive to the sea water temperature as an external environment, while that during phase 2 (November to February) was insensitive to it. The water temperature directly controls the speed of the later phase of spermatogenesis (from spermatocyte to spermatozoon), but it is not able to control the spermatocyte production rate. It is therefore suggested that the water temperature plays an important role in spermatogenesis during phases 3 and 4 by controlling the speed of the later phase of spermatogenesis, and that other environmental factor(s) controlling the spermatocyte production rate are likely to be important to spermatogenesis during phase 2.

INTRODUCTION

Although many detailed studies have been made on the reproductive cycle of echinoderms, especially on that of the echinoids, the strict relationship between the reproductive cycle and environmental changes is still unclear. Based upon a superficial correlation between them, several authors have suggested that the reproductive periodicities might be controlled by environmental factors such as water temperature (Boolootian, 1966; Pearse and Phillips, 1968; Chatlynne, 1969; Pearse, 1969a, b, 1970; Lessios, 1981), salinity (Lessios, 1981), lunar periodicity (Pearse, 1975), tidal change (Pearse, 1972), food supply (Fuji, 1960; Pearse and Giese, 1966; Pearse, 1969a, b; Gonor, 1973), and photoperiod (Holland, 1967; Pearse, 1969a). To date, however, experimental studies to clarify the relationship between the reproductive cycle and these environmental factors have been confined to only a few investigations (Cochran and Engelmann, 1975; Pearse, 1981; Pearse and Eernisse, 1982).

Concerning the reproductive cycle of ophiuroids, several examinations have been published until now, but the effect of the external factors on gametogenesis has never been experimentally confirmed (Patent, 1969; Fenaux, 1970, 1972; Lönning, 1976; Tyler, 1977; Hendler, 1979; Tyler and Gage, 1980, 1982; Gage and Tyler, 1982). In our previous paper, we described the gross features of the annual reproductive cycle of the brittle-star *Amphipholis kochii* (Iwata and Yamashita, 1982), but detailed examinations on the reproductive cycle and the environmental factors still remain. Therefore, a quantitative analysis of the reproductive cycle and an

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experiment to clarify the relationship between the reproductive cycle and the sea water temperature as an environmental factor are carried out in this study.

MATERIALS AND METHODS

The brittle-stars, *Amphipholis kochii,* used in this study were collected between the tidemarks at Abuta on the Pacific coast of south-western Hokkaido, Japan, at monthly intervals from June 1979 through August 1980. Several animals from each collection were fixed in Bouin's solution for ordinary histological preparations as soon as they had been transported to the laboratory, while the remainder were maintained until use.

Calculation for the number of cells in testis

The number of cells in the testis was calculated by the following procedure, based on the method of Holland *et al.* (1975):

1) Assuming that the testis is a solid cylinder with a constant diameter, the testicular volume was calculated according to its length and diameter.

2) Under the light microscope, the histological cross section of a given testis was divided into four regions, each of which is occupied mainly by spermatogonia, spermatocytes, spermatozoa together with spermatids, and others, respectively. The boundary of each region was clearly observable by histological examination (Figs. 1C, D). In order to assess the percentage of each cell population in a cross section, the histological cross section of the testis was projected onto an aluminum foil by means of *camera lucida.* The aluminum foil was cut along the boundary of each cell region and weighed by a chemical balance. The weight of each aluminum foil was divided by the total weight.

3) To estimate the volume of one spermatogonium, spermatocyte or spermatozoon (or spermatid), an arbitrary number of cells in each cell population was projected onto an aluminum foil at a magnification of 920×. The projected foil was then weighed, and its weight was divided by the weight of the standard aluminum foil of 920 mm × 920 mm for conversion of weight to area. The resulting area was divided by the number of the cells projected to yield the area occupied by one cell. From this area, the volume of one cell was calculated on the assumption that the cell was adequately represented as a sphere. The average volume of each cell type after several measurements was as follows:

Spermatogonium: 1.0×10^{6} mm^3

Spermatocyte: 3.6×10^{-7} mm^3

Spermatozoon (Spermatid): 2.4×10^{-8} mm^3

4) In order to calculate the cell number of each cell population from a given testis, the testicular volume was multiplied by the percentage of each cell population and then divided by the volume of the one cell. Three arbitrary testes were measured for an individual, and the average number of cells was used for the data.

To assess the number of the amoeboid cells (the somatic cells in the testis), the foregoing method could not be employed because the locality of these cells in the testis was not cylindrical but very irregular (Fig. 1E). Thus, the area of the amoeboid cell clusters in a cross section was used for estimating the number of the amoeboid cells. It was accomplished as follows: The outline of the clusters of the amoeboid cells in a cross section was projected onto an aluminum foil at a magnification of 194×, and the weight of the foil obtained was divided by the weight of the foil of 194 mm square. The average area after three measurements was used for the data.

Experiment on temperature influence

To examine the influence of sea water temperature on spermatogenesis, some brittle-stars collected in February, April and May were separated into two groups, each containing about five animals; one group, as a control, was kept in the aquarium at the same temperature as that of the sea water at the collection site, while the other group was kept at a higher or lower temperature than that. In this case, the day length at the collection site was roughly maintained in experiment. Eighteen days later, the animals were fixed, and the number of the spermatogenic cells were calculated by the aforementioned procedure.

Autoradiography

The brittle-stars collected during the season from November through June were injected intracoelomically with 5 μCi of ^{3}H-thymidine (specific activity, 42 Ci/m mol: New England Nuclear, Boston). The ^{3}H-thymidine was diluted for use with one part of sea water. The injected animals were kept in the aquaria at various water temperatures including the same temperature at which the samples were collected. At intervals after the injection of 1 h, 1 day, and days 3, 5, 7, 10, 14, 18, 22, 26 and 30, several animals were fixed in Bouin's solution, embedded in paraffin (Tissue Prep; Fischer Scientific Co., New Jersey), and sectioned in pieces 2–3 μm thick. After being deparaffinized, the slides were extracted with cold 2% perchloric acid and dipped in half-diluted NR-M2 nuclear emulsion (Konishiroku Photo Ind., Tokyo) at 45°C; they were then exposed for 10 days at 4°C. The slides were stained with Delafield's hematoxylin and eosin after the photographical development following the application of emulsion.

Electron microscopy

The testes were prefixed with 5% glutaraldehyde in 75% sea water, washed in 150% sea water, and post-fixed with 1% OsO_4 in 75% sea water. The fixed testes were dehydrated with acetone and embedded in Epon 812. Ultrathin sections were cut with glass knives on a Porter-Blum MT-1 ultramicrotome, stained with uranyl acetate and lead citrate, and observed in a JEOL 100S electron microscope.

RESULTS

Histology of the testis

The testes in the disk are little sacs attached to the coelomic wall of the bursae, arranged in a row (Figs. 1A, B). In the testis, zonation of spermatogonia, spermatocytes and spermatozoa together with spermatids can be recognized (Figs. 1C, D). Mature spermatozoa gather to form large clusters in the central portion of the testis (Fig. 1C). The somatic cells in the testis known as amoeboid cells (Iwata and Yamashita, 1982) are usually found during the formation assembly in the sperm mass (Fig. 1E).

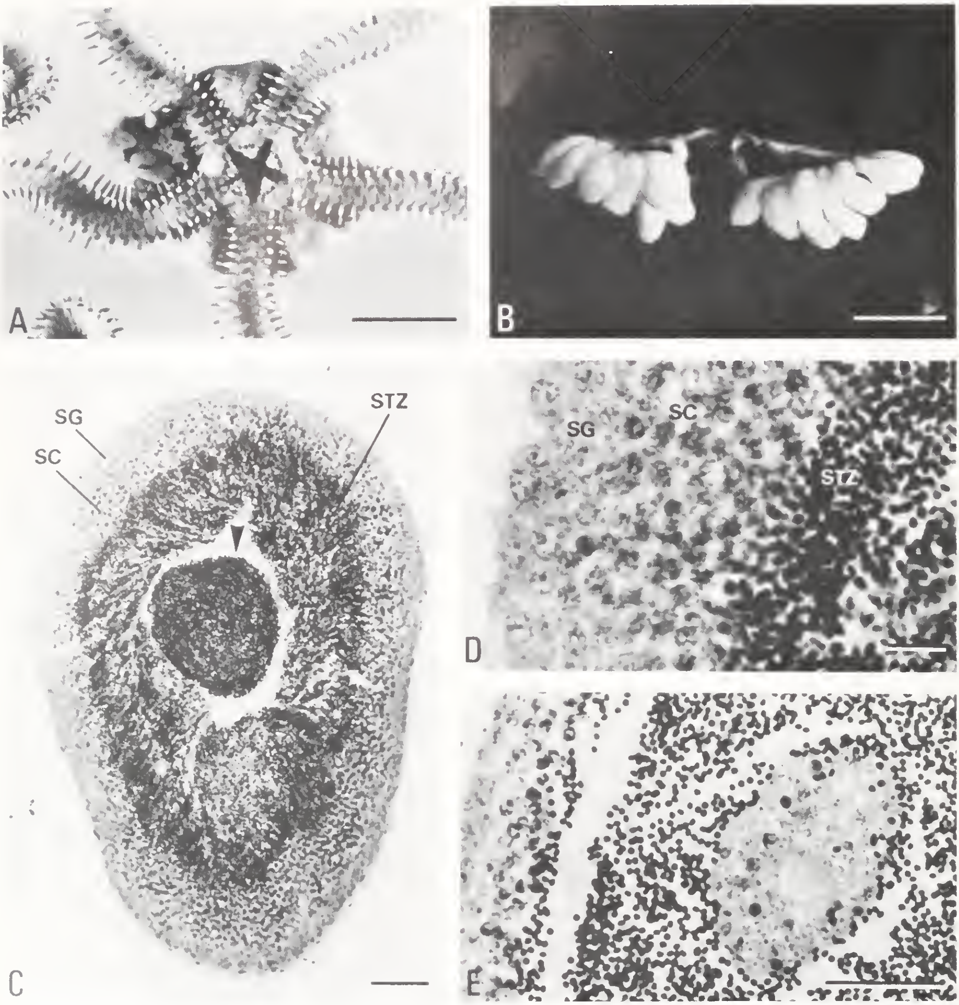

FIGURE 1. A: Photograph of *Amphipholis kochii* from the ventral view, partly dissected to show the attachment and arrangement of the testes. Scale = 5 mm. B: Photograph of the testes isolated from one inter-radial part. Scale = 1 mm. C: Histological cross section of the testis, showing a zonation of spermatogonia (SG), spermatocytes (SC) and spermatozoa together with spermatids (STZ). Scale = 100 μm. D: High magnification of the testis in Figure 1C. From the testicular wall to the inside, spermatogonia (SG), spermatocytes (SC) and spermatozoa together with spermatids (STZ) form zones. Scale = 50 μm. E: The amoeboid cell cluster in the sperm mass. Scale = 50 μm.

Annual cycle of spermatogenesis

Figure 2 gives monthly fluctuations in the number of the spermatogenic cells, the area of the amoeboid cell clusters and the sea water temperature at the collection site. As a whole, the following five phases are distinguishable in the annual testicular cycle.

Phase 1: August to October. Spermatogenesis is arrested, and the spermatocytes are absent. A small number of the relict spermatozoa are ingested by the amoeboid cells forming large clusters.

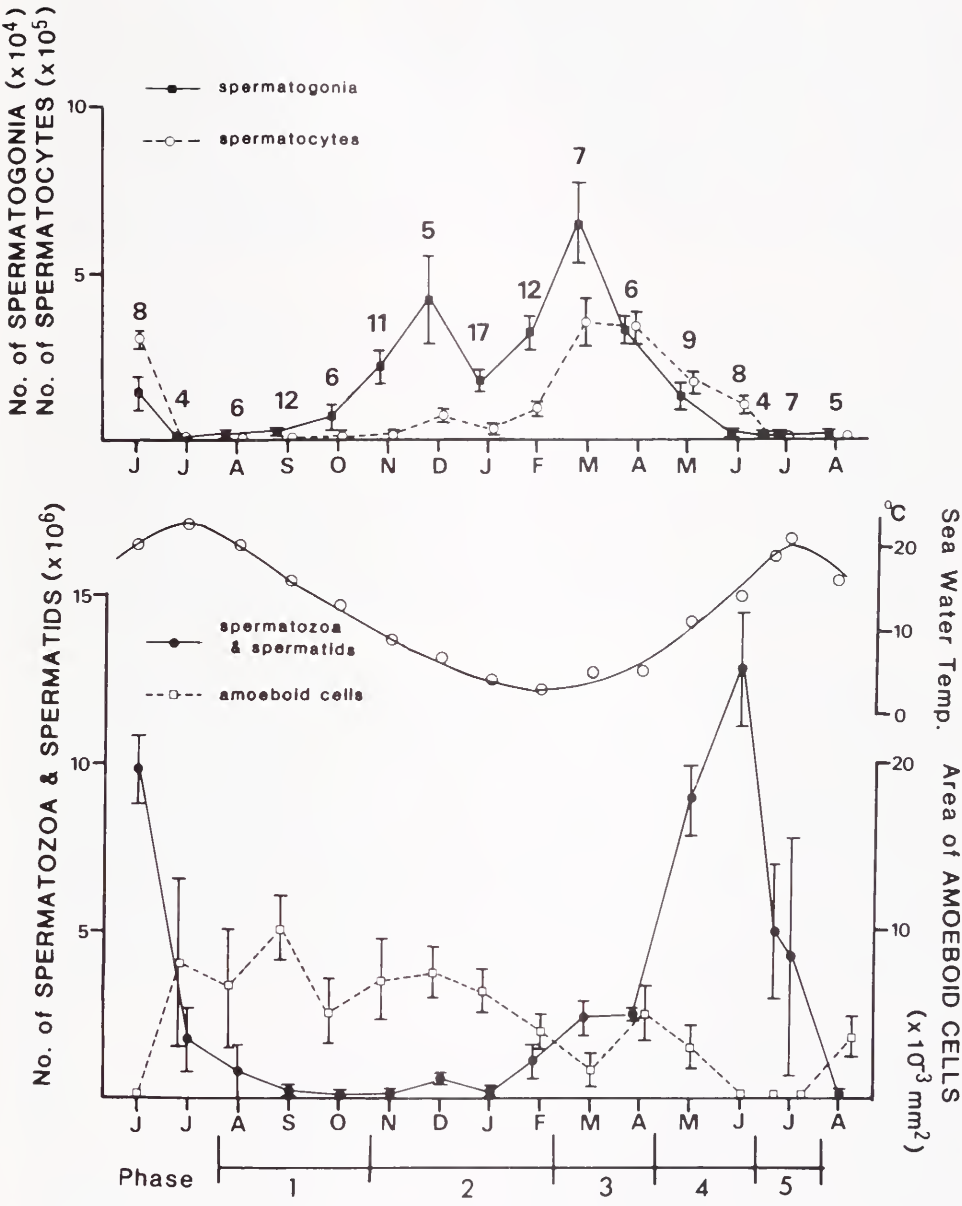

FIGURE 2. Monthly variations in the number of the spermatogenic cells and in the area of the amoeboid cell clusters in *Amphipholis kochii*. Changes of the sea water temperature at the collection site are also indicated. The numbers indicated over the upper graph show the number of the animals used each month. Average ± standard error.

Phase 2: November to February. Spermatogenesis recommences, but the number of the spermatozoa is still very small. The clusters of the amoeboid cells are still large.

Phase 3: March to April. The spermatocytes increase abruptly in number, and the number of the spermatozoa and spermatids becomes larger than in phase 2, but

TABLE I

Number of the spermatocytes and the spermatozoa with spermatids in Amphipholis kochii

	Sea water temperature (°C)	No. of spermatocytes ($\times10^4$)	No. of spermatozoa & spermatids ($\times10^5$)
February	3 (control)	7.3 ± 2.0	10.1 ± 4.6
	13	4.7 ± 1.0	10.8 ± 3.1
April	5 (control)	33.6 ± 5.2	25.3 ± 1.4
	9	29.1 ± 6.7	62.6 ± 14.9
May	11 (control)	9.7 ± 3.5	93.5 ± 12.1
	4	41.4 ± 14.5	70.0 ± 31.5

The animals were kept at a higher or lower temperature than that at the collection site in February, April and May.

Average ± standard error.

much smaller than in phase 4. During this period, the sea water temperature at the collection site is still as low as during phase 2.

Phase 4: May to June. The sea water temperature rises rapidly. The number of the spermatozoa and spermatids increases abruptly, reaching maximum in June.

Phase 5: July. A sudden decrease in the number of the spermatozoa and spermatids occurs as a result of spawning (Iwata and Yamashita, 1982).

Influence of sea water temperature on spermatogenesis

Table I shows the result obtained from the experiment to examine the influence of sea water temperature on spermatogenesis. In February (phase 2), there was no significant difference in the number of the spermatogenic cells between the control and experimental groups. In April (phase 3) and May (phase 4), however, an apparent difference between the two groups was detected. In April, the number of the spermatozoa and spermatids in the animals which had been kept at higher water temperature was significantly larger than that in the control ($P < 0.05$, t-test). In May, the number of the spermatozoa and spermatids at the lower temperature was smaller than that in the control. It was also notable that in May the number of the spermatocytes at lower temperature was much larger than in the control ($P < 0.1$).

These results indicate that the high water temperature accelerates the accumulation of spermatozoa and that the low water temperature inhibits it in phases 3 and 4. In phase 2, however, the water temperature has no influence on the accumulation of spermatozoa.

Duration of spermatogenesis at various temperatures

Figure 3 shows autoradiograms through the testis of the brittle-star *Amphipholis kochii* after the injection of ^{3}H-thymidine. Even 1 h after the injection, the spermatogonia and spermatocytes were heavily labeled, but the spermatids and spermatozoa were not labeled at all (Fig. 3A). Several days after the injection, the spermatids or the spermatozoa became labeled (Figs. 3B, C). This feature shows the occurrence of a possible transformation of the labeled spermatogonia or spermatocytes to the spermatids or spermatozoa, *in vivo*. Finally, the labeled spermatozoa were found in the cytoplasm of the amoeboid cells (Fig. 3D). Under the electron microscope, we could easily detect these spermatozoa ingested by the amoeboid cells after a phagocytic action (Fig. 4). Based upon these results, the duration necessary

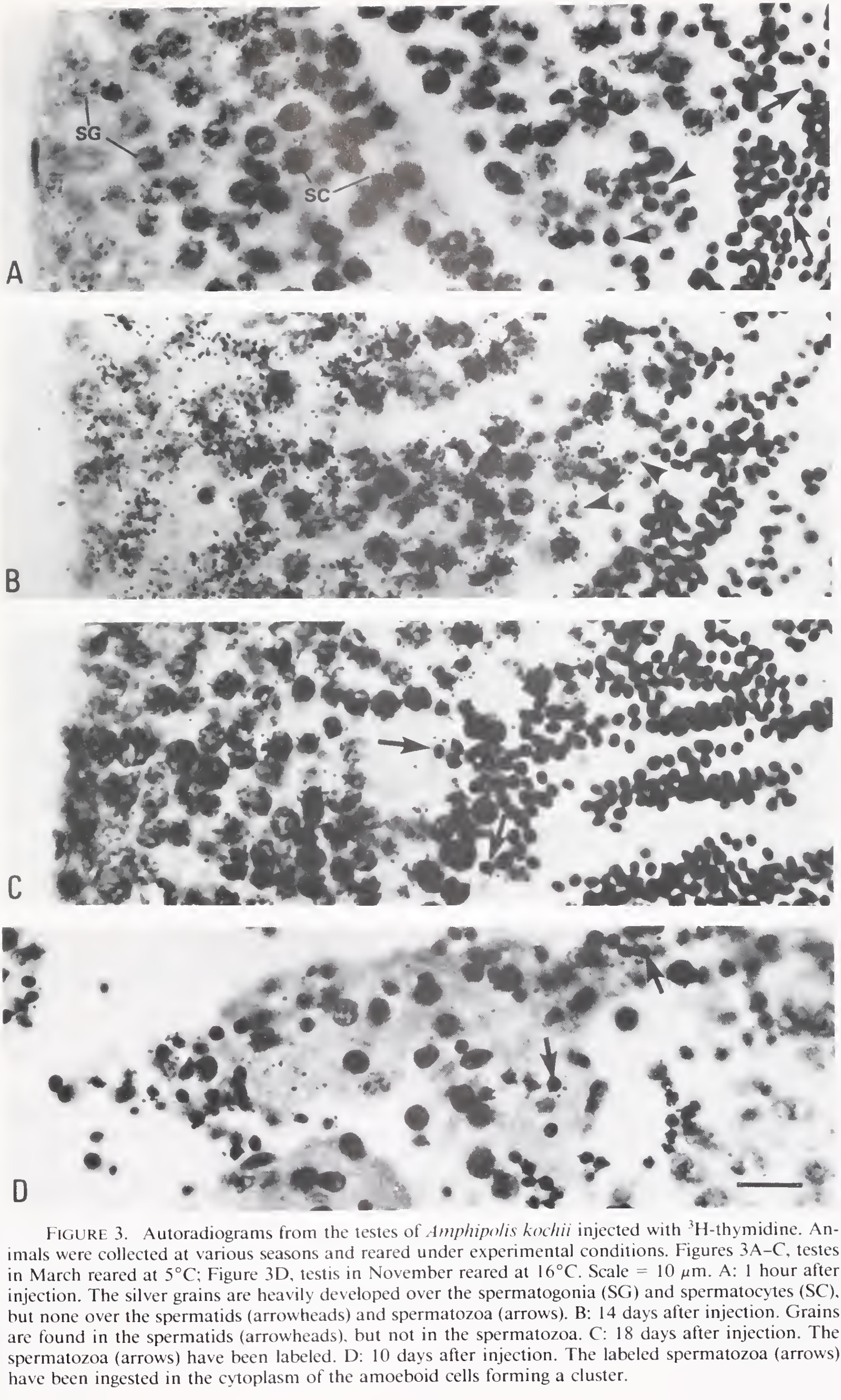

FIGURE 3. Autoradiograms from the testes of *Amphipolis kochii* injected with ^{3}H-thymidine. Animals were collected at various seasons and reared under experimental conditions. Figures 3A–C, testes in March reared at 5°C; Figure 3D, testis in November reared at 16°C. Scale = 10 μm. A: 1 hour after injection. The silver grains are heavily developed over the spermatogonia (SG) and spermatocytes (SC), but none over the spermatids (arrowheads) and spermatozoa (arrows). B: 14 days after injection. Grains are found in the spermatids (arrowheads), but not in the spermatozoa. C: 18 days after injection. The spermatozoa (arrows) have been labeled. D: 10 days after injection. The labeled spermatozoa (arrows) have been ingested in the cytoplasm of the amoeboid cells forming a cluster.

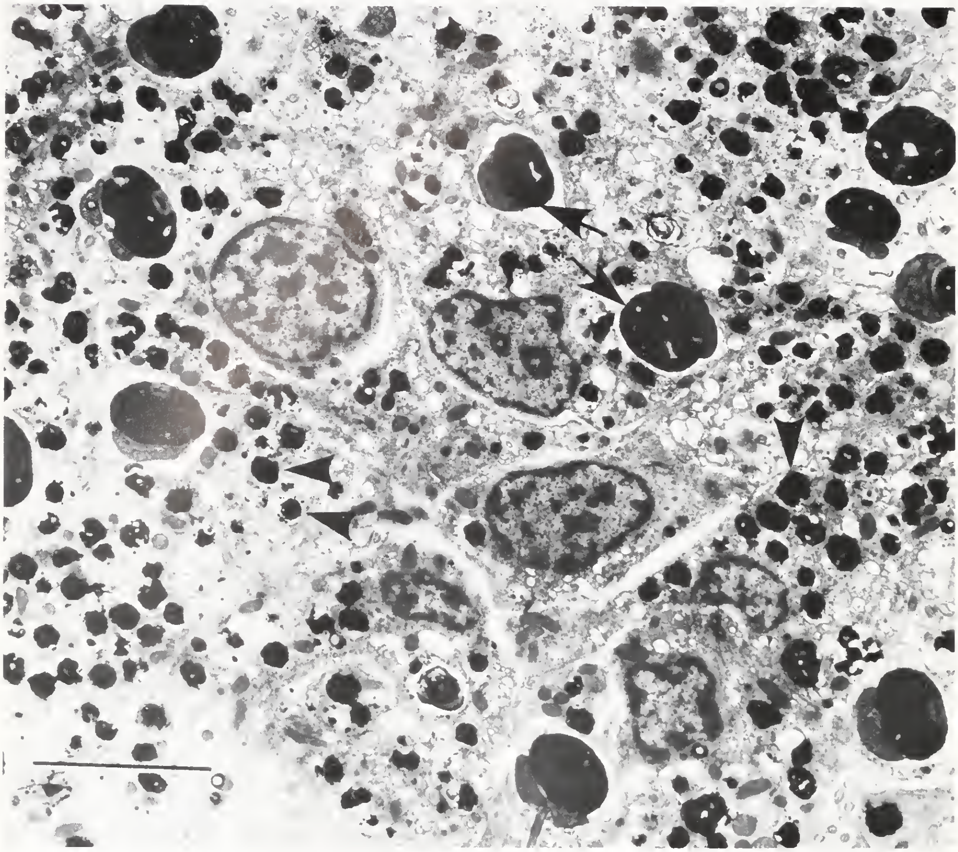

FIGURE 4. Electron micrograph of the amoeboid cells of *Amphipholis kochii* forming a cluster in the sperm mass. Note the mature spermatozoa (arrows) ingested by the amoeboid cells by a phagocytic activity. In the cytoplasm of the amoeboid cells many residual bodies which are probably derived from degenerate spermatozoa are also seen (arrowheads). Scale = 5 μm.

for the transformation from the spermatocyte to the spermatid or to the spermatozoon was determined by detecting the first appearance of the labeled spermatid or spermatozoon in the autoradiograms.

Table II gives the duration of spermatogenesis at various water temperature as well as at the same temperature as that at the collection site. The duration of spermatogenesis faithfully correlated to the water temperature at which the animals had been kept. The same experimental temperature for cultivation yielded the same duration of spermatogenesis irrespective of whether the animals had been collected at different months. In order to demonstrate the relationship between the duration of spermatogenesis and the water temperature more closely, the durations obtained in each month in Table II were plotted against the water temperature (Fig. 5). As the water temperature became higher, the duration of spermatogenesis became shorter, and *vice versa.* A close relationship between the water temperature and the duration of spermatogenesis was more apparent in the early stage of spermatogenesis, from spermatocyte to spermatid, than in the later stage, from spermatid to spermatozoon or spermiogenesis.

TABLE II

Duration of spermatogenesis in Amphipholis kochii *at various water temperatures*

	Nov		Dec	Jan	Feb		Mar		Apr		May		Jun	
Sea water temperature (°C) (collection site)	9		7	4	3		5		5		11		14	19
Sea water temperature (°C) (experiment)	9	16	7	4	3	13	5	18	5	9	11	4	14	19
Duration of spermatogenesis (days)														
spermatocyte—spermatid	7	5	10	18	22	5	14	3	14	7	7	22	5	3
spermatocyte—spermatozoa	14	7	14	22	30	10	18	5	18	14	10	30	7	5
spermatid—spermatozoa	7	2	4	4	8	5	4	2	4	7	3	8	2	2

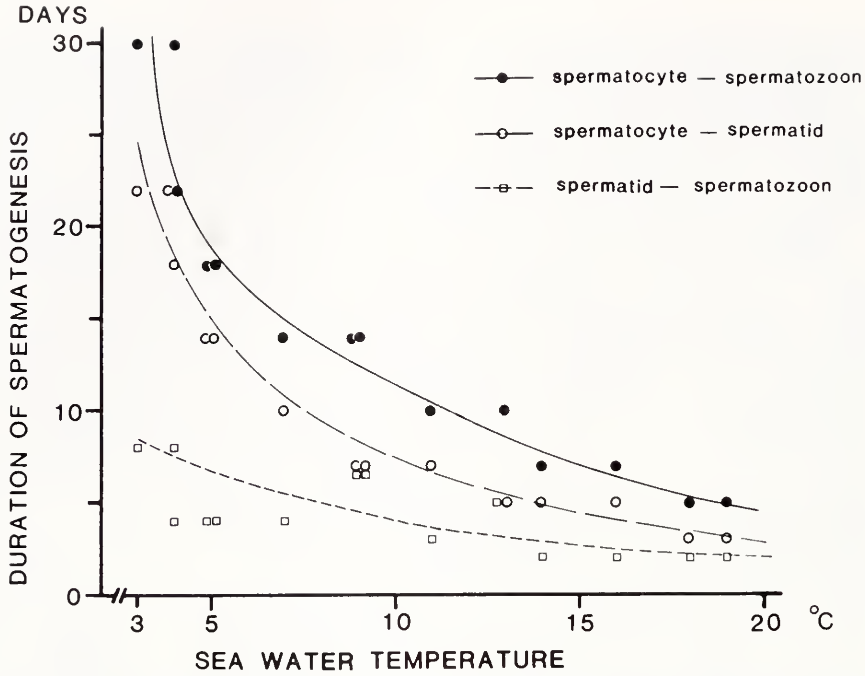

FIGURE 5. Correlation between the duration of spermatogenesis of *Amphipholis kochii* and the sea water temperature. The animals were reared at various experimental temperatures and the duration of spermatogenesis in each experiment was then measured.

Seasonal changes in the spermatocyte production rate (SPR)

To estimate the SPR per day throughout each month, the number of the spermatocytes (Fig. 2) was divided by the duration of the spermatocytic stage (the duration from spermatocyte to spermatid, Table II). Table III shows the seasonal changes in the SPR. It was apparent that the seasonal changes in the SPR had two distinct periods, one from November to February (phase 2) and the other from March to June (phases 3 and 4). The SPR in phases 3 and 4 was about six times higher than that in phase 2. The SPR was high in phase 3 though the sea water temperature was noticeably low at this season (Fig. 2). In phase 2, however, the SPR

TABLE III

The spermatocyte production rate in Amphipholis kochii *during the season from November to June*

	Phase 2				Phase 3		Phase 4	
	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
No. of spermatocytes ($\times 10^4$) (N)	1.4	7.2	3.3	9.2	34.6	33.6	17.3	9.7
Duration of spermatocytic stage (days) (D)	7	10	18	22	14	14	7	5
N/D	0.2	0.7	0.2	0.4	2.5	2.4	2.5	1.9
Spermatocyte production rate (per day)	1.0	3.5	1.0	2.0	12.5	12.0	12.5	9.5

The rate in each month is represented as a ratio to the rate obtained in November.

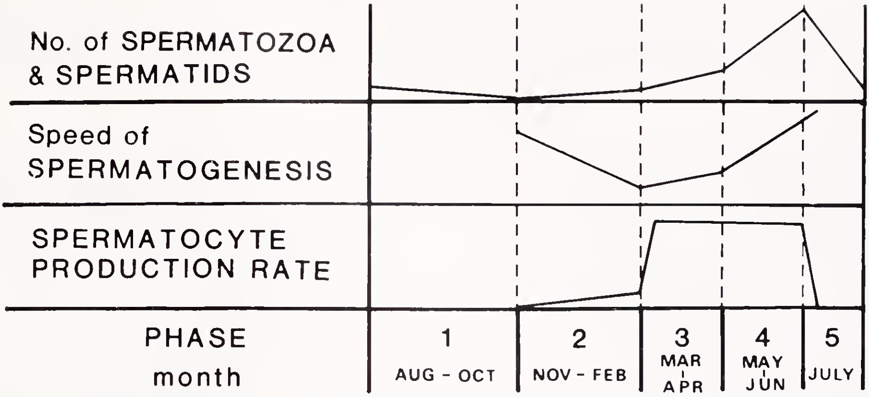

FIGURE 6. Seasonal tendencies in the number of the spermatozoa and spermatids, the speed of spermatogenesis and the spermatocyte production rate, summarized from Figure 3 and Tables II and III.

was very low even at almost the same or higher water temperature than that in phase 3 (Fig. 2). These results seemed to indicate clearly that the water temperature was not able to control the SPR.

DISCUSSION

The present study demonstrates a close correlation between the duration of spermatogenesis of the brittle-star *Amphipholis kochii* and the water temperature as an external environment, both under normal and experimental conditions. Seasonal changes in the annual testicular cycle and the factors controlling spermatogenesis are summarized in Figure 6. Based upon Figure 6, we suggest the following control mechanisms for spermatogenesis during phases 2 to 4. Phase 2: The sluggish accumulation of the spermatozoa in this phase is probably due to the low spermatocyte production rate. In this phase, the water temperature influences neither the accumulation of the spermatozoa nor the spermatocyte production rate (Tables I and III). The water temperature should not therefore contribute to the control of spermatogenesis during this phase. Phase 3: Owing to the high spermatocyte production rate, the accumulation of the spermatozoa becomes active, but it is slower than that in the next phase. This situation is presumably due to the slow speed of spermatogenesis resulting from the low sea water temperature at this season (Figs. 2, 5). This explanation is consistent with the experimental results that the number of the spermatozoa grew larger when the animals were kept during phase 3 at a higher water temperature and that it decreased during phase 4 when the animals were kept at a lower temperature (Table I). The increase of the spermatocytes in the animals kept at a lower temperature in phase 4 (Table I) is therefore probably due to the slow speed of spermatogenesis caused by the low water temperature. Phase 4: The spermatocyte production rate is high and the speed of spermatogenesis is fast owing to the high sea water temperature, resulting in a rapid accumulation of the spermatozoa.

With respect to the water temperature as an external factor on the control of the annual reproductive cycle, the testicular cycle of *Amphipholis kochii* can be divided into two distinct periods: the temperature-insensitive period during the season from November to February (phase 2) and the temperature-sensitive period from March to June (phases 3 and 4). The water temperature plays an important role as a key factor on spermatogenesis during phases 3 and 4, while other environmental factors such as photoperiod or food supply are likely to be key factor(s) on spermatogenesis during phase 2. Further studies to examine the effect of day length or food supply on spermatogenesis, especially during phase 2, seem to be necessary.

Concerning the seasonal changes in the speed of spermatogenesis, Holland and Giese (1965) carried out an autoradiographic study on the testis of the echinoid *Strongylocentrotus purpuratus*, and obtained the result that the duration of spermatogenesis remained constant throughout the annual cycle. An explanation for Holland and Giese's result, in contrast with that of this study, might be that they measured the duration of spermatogenesis at relatively constant water temperatures throughout the annual cycle.

The possible participation of the somatic cells in the control of the spermatozoa production rate cannot be ignored. By means of an autoradiographic investigation, Holland and Giese (1965) revealed the ingestion of newly-formed spermatozoa by the somatic cells in the testis of the echinoid. The present study also demonstrates that the amoeboid cells (the somatic cells) in the testis of *Amphipholis kochii* are able to ingest the newly-formed spermatozoa as well as the relict ones by phagocytic action (Fig. 4). Judging from the presence of large clusters of amoeboid cells during phase 2, we might be allowed to assume that the phagocytic activity of the amoeboid cells upon the newly-formed spermatozoa contributes partly to a slow spermatozoa accumulation in phase 2.

The duration of spermatogenesis has been measured in various animal phyla, and it is certain that marine invertebrates have a relatively high speed of spermatogenesis in comparison with mammals and insects (see Table 9 in Roosen-Runge, 1977). The present measurement also reveals that the spermatogenesis of the brittle-star is of a relatively short duration. This short duration may affect the mode of spermatogenesis. For example, the spermatogenesis of the present species is very peculiar in that the acrosome formation is initiated in the spermatogonium (Yamashita and Iwata, 1983). This prococious production of the acrosome might be related to the short duration of spermatogenesis insufficient for the production of acrosomal material during spermiogenesis. The similar phenomenon has also been reported in the mussel, in which the acrosome formation is initiated in the spermatogonium (Longo and Dornfeld, 1967) and the duration of spermatogenesis is relatively short (Kelley *et al.*, 1982). On the other hand, it is known that the sea urchin has a short period of spermatogenesis (Holland and Giese, 1965) but that the acrosome formation is initiated in the spermatid (Longo and Anderson, 1969). This contrasting situation between the sea urchin, brittle-star, and mussel may be explained by the fact that the acrosome of the sea urchin is small when compared with that of the brittle-star and mussel (Dan, 1967; Yamashita and Iwata, 1983). Although the spermatogenesis of the sea urchin is of a short duration, it may be sufficient for the production of such a small acrosome during the spermatid stage.

It is very interesting to note that the duration of spermiogenesis appears to be independent of temperature (Fig 5). In this respect, Egami and Hyodo-Taguchi (1967) reported that the duration of spermiogenesis in the fish *Oryzias latipes* at 25°C and 15°C showed no significant difference. These observations allow us to suggest that spermiogenesis mainly depends on internal factors.

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A PROPOSED DUAL ROLE OF ODOR IN FORAGING BY THE CALIFORNIA SPINY LOBSTER, *PANULIRUS INTERRUPTUS* (RANDALL)

RICHARD K. ZIMMER-FAUST* AND JAMES F. CASE**

Department of Biological Sciences and the Marine Sciences Institute,
University of California, Santa Barbara, California 93106

ABSTRACT

A dual influence of odor on foraging is proposed for *Panulirus interruptus*, on the basis of laboratory and field tests using abalone muscle effluence as a stimulant. Food search consisted of three major components: detection (increased antennule flicking), locomotion, and non-locomotor probing by pereiopod dactyls. Detection occurred at lower concentrations (10^{-8} to 10^{-10} g/l) and was initiated before probing and locomotion in laboratory tests. Probing occurred at concentrations $\geq 10^{-6}$ g/l and was initiated before locomotion. Locomotion was limited to higher concentrations ($\geq 10^{-4}$ g/l) and its induction frequently followed introduction of an effective chemical stimulus by 60 s or longer. The response hierarchy in *Panulirus* indicates that concentrated chemical stimuli may initiate only local searches for food.

Traps were baited with abalone muscle for field experiments. Effective effluent concentrations in immediate trap environments were estimated by a three-dimensional Fickian diffusion model. The minimum concentration attracting lobsters was estimated to be nearly identical to the laboratory-determined threshold for detection, 4–6 log units lower than the threshold for induction of locomotion. Lobsters were captured in traps primarily at night, the period of greatest normal, endogenously initiated activity. Consequently, low concentrations may act by modifying behavior of animals already aroused, rather than by initiating foraging or feeding from the quiescent state.

INTRODUCTION

Chemical stimuli induce food search in decapod crustacea frequently in the absence of other sensory cues (McLeese, 1970, 1973a; Shelton and Mackie, 1971; Mackie and Shelton, 1972; Mackie, 1973; Carr and Gurin, 1975; Hindley, 1975). Laboratory experiments have shown visual and tactile prey stimuli often are without effect, while odors released from prey cause marked food searching responses (Derby and Atema, 1981; Schembri, 1981; Zimmer-Faust and Case, 1982a). Field experiments have recently demonstrated that the lobster, *Panulirus interruptus*, and the crabs, *Cancer antennarius* and *Loxorhyncus grandis*, discriminate among odors of adjacently positioned foods and that chemical stimuli direct the movements of lobsters both towards and away from potential food items (Zimmer-Faust and Case, 1982b).

Chemical stimuli are commonly believed to influence decapod foraging by triggering distance chemoreception (Hazlett, 1971a, b; McLeese, 1973b; Ache *et al.*,

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* Present address: Department of Zoology, University of Queensland, St. Lucia, Brisbane 4067, Queensland, Australia.

** Address for reprint requests.

1978; Pearson *et al.*, 1979; Reeder and Ache, 1980). According to this model, dilute attractant concentrations are thought to stimulate highly sensitive chemoreceptors, thus causing locomotor responses towards distant targets. Chemical concentration is assumed to be an important cue relating distance to food, and dilute attractant concentrations are presumed to be interpreted by animals as originating from distant food sources. Highly sensitive chemoreceptors have been identified on the antennules of the Florida spiny lobster, *Panulirus argus* (Fuzessery, 1978; Thompson and Ache, 1980), and extremely low behavioral thresholds for detection have been observed in other decapods (Pearson and Olla, 1977; Price and Ache, 1977; Pearson *et al.*, 1979); however, it has yet to be demonstrated that very low stimulant concentrations can induce locomotion. Decapods are presently known to initiate locomotion at concentrations of 10^{-6} to $\geq 10^{-2}$ g/l, when responding to various tissue extracts (Mackie and Shelton, 1972; Mackie, 1973; McLeese, 1973a; Pearson *et al.*, 1979). Orientation to chemical gradients has been demonstrated for the lobsters, *Homarus americanus* and *Panulirus argus*, but exclusively in response to extract concentrations $\geq 10^{-3}$ g/l (McLeese, 1973b; Reeder and Ache, 1980). These results suggest that chemical stimuli influence local searches for food, where high stimulant concentrations are maintained, but offer little evidence concerning the effect of chemical stimuli on distant foraging.

The present study was conducted to determine the influence of odor on foraging behavior of the California spiny lobster, *Panulirus interruptus* (Randall). Laboratory and field estimates were made for concentrations of effluence released from abalone (*Haliotis* spp.) muscle that would be effective in stimulating lobsters. Results showed that high concentrations initiated behavioral patterns of local searches for food. Low concentrations appeared to modify, but not initiate, distant searches for food. Evidence showed that the action of chemical stimuli was limited to the near vicinities of food, and that long-range chemosensory responses were unlikely to be manifested by *Panulirus* in its naturally turbulent habitat.

Materials and Methods

Selection of abalone muscle effluence as chemical stimulant

In the selection of a chemical stimulant for this investigation, it was essential that it be capable of inducing food search in lobsters under both laboratory and field conditions. Ideally, this stimulant should consist of one or only a few readily detectable compounds. Low molecular weight substances, particularly amino and organic acids, were desirable constituents for the stimulant because standard chemical assays exist for these compounds, and laboratory studies show amino and organic acids to be highly effective in stimulating food search among decapod crustacea (McLeese, 1970; Shelton and Mackie, 1971; Mackie, 1973; Hindley, 1975; Carr, 1978). Unfortunately, release of single amino and organic acids has failed to attract animals consistently in field experiments (Allen *et al.*, 1975; Ache *et al.*, 1978).

Preliminary to the present investigation, we performed tests involving release of single amino and organic acids and a five-component amino-organic acid mixture from polyacrylamide gels. These solutions failed to attract lobsters, as in previous studies, even though they were highly stimulatory in laboratory tests. Gels releasing a sea water extract of abalone muscle successfully attracted lobsters. Effluence from abalone muscle was, therefore, selected for use in present experiments. While not providing a completely defined chemical stimulus, abalone muscle produces an attracting odor in both laboratory and field experiments (Zimmer-Faust and Michel, 1980; Zimmer-Faust and Case, 1982b). Abalone muscle is available to *Panulirus* as carrion in the natural habitat. Stimulatory molecules released by abalone muscle are identified as peptides and polypeptides of widely ranging molecular weights (Zimmer-Faust and Michel, 1980).

Laboratory experiments: collection and maintenance of animals

Lobsters were captured by hand (SCUBA) or by trap at More Mesa reef, the habitat used for our field trapping experiments, 4 km east of the U.C. Santa Barbara campus. Captured animals were brought immediately to the laboratory and held in groups of 10 individuals in 3,000 l aquaria, for 10–14 days prior to experiments. Excess shelters were provided. A continuous, single-pass sea water flow (5 μm filtered) in each holding tank maintained aeration and ambient sea temperatures (16–19°C). All incoming animals were marked using the tattoo method of Kuris (1971), and carapace length, sex, and reproductive status were recorded for each animal. Only adult intermoult animals of mean carapace length, 66 mm (±3 SD), were used in experiments. A 12:12 D:L cycle (light on: 0600–1800 h) was maintained throughout holding and experimental periods, and all tests were conducted during the 1900–2400 h period. Animals were fed abalone muscle, mackerel muscle, and opened mussels *ad libitum* on alternate days and deprived of food for 24 h before testing.

Test apparatus

Animals were individually tested for responses to chemical solutions in rectangular aquaria, 30 × 30 × 13 cm constructed so as to allow precise control of stimulus flow characteristics. Opaque blinds around each aquarium allowed observation without disturbing test animals. Dim lighting was provided by 25-watt red incandescent bulbs in diffusing housings placed 50 cm above aquaria. Lighting was completely confined by the blinds and the surrounding laboratory was maintained in darkness to reduce disturbances to test animals. Responses of animals in these experiments were nearly identical to those of animals tested later, both during the day and at night, using ambient light intensities measured at the collection site (T. Frank, Dept. of Biology, U.C. Santa Barbara, pers. comm.). Light intensities used in present experiments, therefore, appear not to influence behavioral thresholds.

Sea water entered each test aquarium by a delivery system maintained under constant hydrostatic pressure. Polyethylene tubing carried a primary sea water flow (980 ml/min) from a head-tank to a Nalgene adapter, where it was then delivered to each test aquarium at the centerpoint, 0.5 cm below the water surface. A valve in each delivery tube enabled fine adjustments of water flow. A secondary flow to each aquarium, serving as a loop injector for stimulants, was carried by polyethylene tubing (120 ml/min) from a head-tank to a stimulus reservoir, before it joined with the primary flow at a Nalgene adapter. Stimulant was introduced (10 ml/7 s) by opening a three-way valve that connected the stimulus reservoir to the secondary flow system, and flow was uninterrupted during stimulant introductions. A fitting in each Nalgene adapter eliminated back-pressure on secondary flow, causing sea water and stimulants to pass from secondary to primary flow before entering aquaria.

Fluorescein dye (10 ml/7 s) was used to estimate stimulus dilutions. A Masterflex peristaltic pump (Model 7568) coupled to 1.57 mm ID polyethylene tubing continuously evacuated sea water from experimental aquaria (2 ml/min), beginning 180 s prior to and continuing for 180 s following an introduction of dye. Evacuated water was continuously pumped through a Turner fluorometer (Model 111) having Whatman 2A and 47B excitation filters and a Whatman 2A-12 emission filter. Fluorometer output was recorded on a chart recorder. Standards were prepared from fluorescein dye and calibration curves were generated using the fluorometer. Two dye injections were performed for each aquarium, while assay water was evacuated from tubing attached to the basal segments of antennules of unrestrained lobsters. Dye tests indicated that stimulant delivery and mixing was uniform among the test aquaria.

Preparation of test solutions

Stimulants were prepared by collecting abalone muscle effluence (AME) from tissues as they were readied for field experiments. To standardize chemical composition, only one collection was made from which all stimulants were produced. Following collection, AME was filtered (Millepore 0.45 μm membrane) and vortex-stirred to produce a homogeneous mixture. Aliquots of 1 ml were placed in test tubes, capped, and frozen.

Stimulant concentrations were determined by removing fluids (AME) from randomly selected test tubes and heating at 40°C until stable dry weights were attained. Concentrations were determined as the mean dry weight of material (g) per fluid volume AME (ml). These procedures controlled for possible discontinuities in the collection and subsampling of AME. Aliquots were considered homogeneous if nearly identical dry weights were attained for each sample.

Test procedures and threshold determinations

Testing consisted of a random presentation of a test or control stimulus to an animal in a randomly selected aquarium. Animals were actually tested only if inactive, so that procedures would conform to those of previous investigations (*e.g.* McLeese, 1970; Mackie and Shelton, 1972; Mackie, 1973; Pearson and Olla, 1977; Ache *et al.*, 1978; Pearson *et al.*, 1979; Derby and Atema, 1981). Identical solutions were never repetitively introduced to the same animal during the experiment. Only one trial was performed per animal on any single day. At the conclusion of the experiment, each of the 6 different test and control stimulants had been introduced to the same 25 animals.

All trials were conducted by one person who did not know the composition of solutions being tested. Animals were put in experimental aquaria 90–120 min prior to testing and settled within 30–40 min. Observations of behavior were initiated 1 min before stimulus introduction and continued for 3 min after introduction. Verbal descriptions were recorded using a portable tape recorder and were later transcribed and analyzed. Movements associated with locomotion and with non-locomotor probing by pereiopod dactyls were selected for observation because of their obvious roles in searching for distant and local food items, respectively. Antennule flicking was also observed because electrophysiological and behavioral experiments show increased rates of flicking commonly associated with the detection of chemical stimuli (Snow, 1973; Pearson and Olla, 1977; Price and Ache, 1977; Pearson *et al.*, 1979; Schmitt and Ache, 1979). Threshold concentration for each component behavior was defined as the lowest tested concentration to which the proportion of responding animals was significantly greater ($P < 0.05$) than the proportion responding to filtered sea water. Significance levels were determined using the Fisher Exact Test.

Field experiments: general procedures

Tests were performed to estimate concentrations of abalone muscle effluence in the immediate vicinities of traps capable of attracting lobsters. All experiments were conducted at More Mesa reef, where 8 stations were permanently buoyed in 2–4 m water depth. Each station was separated by a minimum of 15 m. Elliptically shaped polyethylene mesh traps were used, 100 cm × 79 cm × 30.5 cm (Fathoms Plus, San Diego, CA). Since repetitive trapping was conducted at a restricted number of sites, the possibility of immediate recapture and trap habituation existed. To monitor this possibility, captured animals were tagged with return addressed, serially numbered anchor tab tags. All tagged animals were released at the point of capture.

Comparisons among capture rates of lobsters are more rigorous if water conditions are known. For this reason, we used the dissolution rate of cast calcium sulfate blocks as a general measure of wave and surge conditions (Doty and Doty, 1973). Twenty-five g cubes were formulated as described by Zimmer-Faust and Case (1982b). On each test date, 8–16 cubes were placed in labeled Vexar bags (0.32 cm mesh) and exposed both in field and laboratory for 24 h. In the field, cubes were placed in a trap having closed entries, located at the centerpoint of the trap matrix. In the laboratory, each cube was placed in a separate 12 l sea water bath without water motion and held at ambient sea temperature. A relative water motion index was created. An index value of zero indicated that the dissolution rate of field-placed cubes was equal to that of cubes positioned in water baths and that no water motion had occurred (Zimmer-Faust and Case, 1982b). A value of one meant that field cubes had totally dissolved. Index values of zero and one were never attained.

Preparation of tissues

Prior to field use, abalone muscle was chopped in a Furnas Buffalo Chopper (Model JP 5) at low speed for 30–60 s and reduced to 1–2 cm cubes. Chopping was deemed essential to increase tissue surface area exposed to sea water and thus to improve the catch. Lobsters were previously found unattracted to live-prey baited traps (Zimmer-Faust and Case, 1982b). Immediately following chopping, tissue was flash frozen at −70°C. Twenty-four hours prior to use the bait was thawed to 0°C and apportioned into labeled 0.32 cm mesh Vexar bags. Rittschof (1980) has shown action of freeze-thawing on chopped molluscan tissues to be similar to that of natural autolytic degradation. Tissue-filled bait bags were placed in a 2.5 cm mesh Vexar box, removed to a temperature-humidity controlled room (temp: 10°C; rel hum: 50%) and allowed to thaw for an additional 12 h. Wet weights were determined and tissues were immediately used in trapping. Effluence was collected as thawing occurred, and used as the stimulant in present laboratory experiments. Vexar bags provided a precise means of placement and later removal of specified amounts of tissues from traps. Bags were inaccessable to captured animals, guaranteeing that only waterborne substances were available as chemical cues.

Test procedures

Seven different quantities of abalone muscle, from 3–372 g, were tested for effectiveness in capturing lobsters. Within any two-day period a single quantity of abalone was tested against empty Vexar packets, using bait reversal procedures (Zimmer-Faust and Case, 1982b). At each of the 8 stations, two traps were paired 1–2 m apart. On the first day, tissue was placed in one trap while an empty Vexar packet was placed in the alternative trap. The first day allocation of tissue was at random and traps were raised after 24 h. On the second day, traps were identically placed with bait positions reversed.

Tests were conducted for a total of 14 two-day periods. During the initial seven two-day periods, abalone mass was successively halved and presented in a descending order. In the final seven two-day periods, quantities of abalone were tested in a randomized order. Data were initially analyzed using Wilcoxon paired comparison tests. In the present report, data were summarized as the total number of animals captured per bait treatment, and exclusive use was made of Chi-square tests for heterogeneity. Significance levels determined by Chi-square were identical to those determined by Wilcoxon.

Rates of effluence

Tissues were placed in traps closed to entry and positioned in the study area during field trapping experiments. Wet weights were determined for these tissues before and after removal from traps, following 0, 1, 2, 4, 8, 18 or 24 h. Dry weights were subsequently determined and wet weight-dry weight regressions were generated for tissues not field exposed. This enabled calculation of both wet and dry weight losses for field-exposed tissues. Field exposure durations were carefully recorded to the minute, and rates were determined as derivatives relating the instantaneous reduction in bait mass to field exposure duration. Rates were later used to estimate effluence concentrations in immediate trap environments since direct assay was impossible.

RESULTS

Laboratory experiments

Peak concentrations of dye attained in test aquaria were 1.02×10^{-3} ($\pm 0.13 \times 10^{-3}$ SD) times original injected concentrations. Peaks occurred 19 s (±4 SD) after

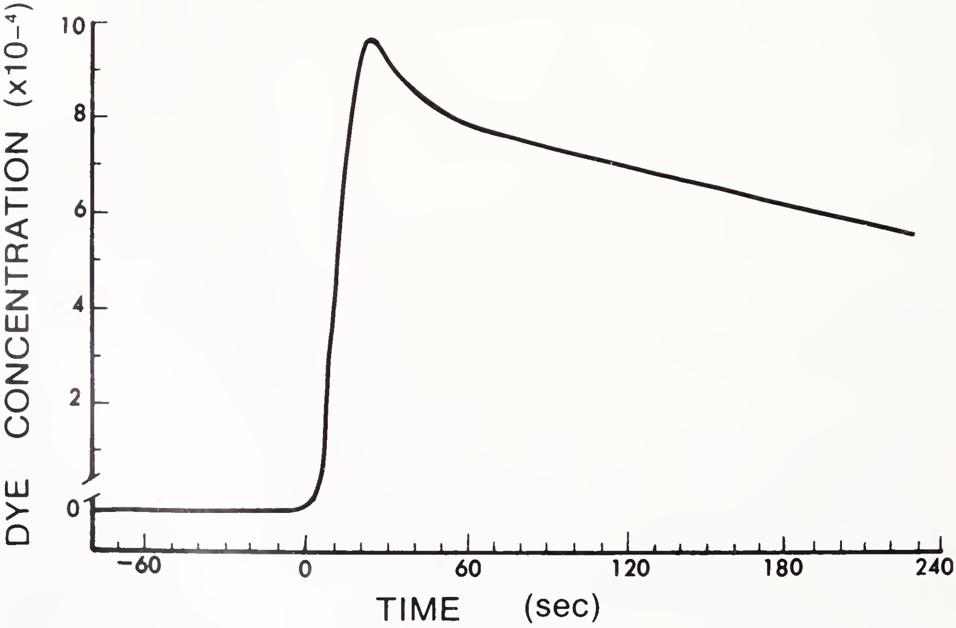

FIGURE 1. A record showing the flow characteristics of a test aquarium following dye introduction (10 ml/7 s). The maximum concentration achieved is 9.7×10^{-4} times the original injected concentration. Time equals zero is defined as the initiation of dye input. Water was evacuated (2 ml/min) from tubing attached to the basal segments of antennules of lobsters, and dye concentrations were continuously monitored using a fluorometer.

TABLE I

Initiation of behavior following stimulus introduction[a]

Behavioral component	Time of initiation (s)[b]	Ranked order of initiation		
	Median (range)	First	Second	Third
Detection (increased antennule flicking)	2(1–15)	19	7	0
Probing	3(2–121)	7	18	1
Locomotion	45(10–176)	0	1	25

[a] Includes only those 26 trials in which all 3 behavioral components were observed.
[b] Time equals zero defined as initial stimulus input.

initial dye input and decayed to 68% of maximum within the 180 s observation period (Fig. 1). Flow characteristics were nearly identical for each experimental aquarium, with no significant differences occurring among peak concentrations, latencies, or decays (One-way ANOVA, with replicates: d.f. = 7/8, $F \leq 1.89$, $P \gg 0.10$, all comparisons).

Concentrations of sampled AME aliquots were equivalent and ranged from 9.80×10^{-2} to 9.89×10^{-2} g/ml, with a mean of 9.83×10^{-2} g/ml ($\pm 0.03 \times 10^{-2}$ SD). This indicated that the collection and subsampling of AME was homogeneous. Average concentrations of AME, in contact with test animals were calculated by multiplying 9.83×10^{-2} g/ml times serial dilutions (10^{-1}, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9}), then multiplying again by the mean stimulus dilution (1.02×10^{-3}). This gave average concentrations contacting the animals of 1.00×10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10} g/l. Hereafter, concentrations cited are those contacting animals.

Response hierarchy

The behavioral components of food search were exhibited in a linear hierarchy. Increased antennule flicking and probing of legs occurred immediately upon the introduction of an effective chemical stimulus, while locomotion was often delayed for 60 s or longer (Table I). When all three behavioral components were observed in the same trial, flicking was initiated before leg probing, which in turn nearly always preceded locomotion. Threshold concentrations for antennule flicking, leg probing, and locomotion were 10^{-8}, 10^{-6}, and 10^{-4} g/l, respectively (Table II). Thresholds appeared representative for leg probing and locomotion, but a large proportion of animals responded to 10^{-10} g/l by increasing antennule flicking. Consequently, a threshold of 10^{-8} to 10^{-10} g/l seems probable for this behavior.

Field experiments

Recapture rates were low for *Panulirus* during field experiments. Only 2.1% of all tagged animals were recaptured. Lobsters recaptured during ensuing commercial seasons increased the total recapture to 10%. This indicated that tags were successfully retained by lobsters, and that near non-replicate capture occurred in our experiments.

Traps having 46–372 g abalone were equally effective in capturing lobsters, while a significant decrease in capture occurred with less than 20 g abalone per trap (Table III). Quantities as low as 7.20 g/trap were effective in capturing animals. Abalone mass decreased linearly as a function of field exposure duration over the first 24 h,

TABLE II

Number of animals responding to stimulants

	Behavioral component			
Concentration (gm/l)	Detection (Increased antennule flicking)	Probing	Locomotion	Sample size
10^{-2}	25***	23***	16***	25
10^{-4}	25***	14*	6*	25
10^{-6}	20**	13*	2	25
10^{-8}	24***	9	3	25
10^{-10}	16	5	1	25
0 (Sea water controls)	10	6	0	25

* The difference between the proportion of animals responding to test as opposed to control solutions is significant (Fisher Exact Test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

meaning that the rate of effluence was constant for this period (Table IV). A sigmoidal log-linear relationship was observed between capture efficiency and rate of effluence, and rates differing less than one log unit in magnitude caused widely varied captures (Fig. 2). This indicated that effluent releases, hence the effluent concentrations in immediate trap environments, were critical in the attraction of lobsters. Effluences of 0.04 g/h caught low but significant numbers of lobsters, while effluences of ≥0.32 g/h produced maximum asymptotic capture rates.

The possibility existed in field experiments that microbial action influenced the stimulatory capacity of abalone muscle, making field and laboratory stimulants not comparable. To explore this possibility, we prepared an extract from lyophilized

TABLE III

Lobsters captured in trapping experiments using abalone muscle as bait

	(abalone-baited vs unbaited traps)[b]					Rate of effluence (g/h)		
Initial mass[a] (g wet weight) x̄ ± se	Day		Day					Water flow[c] index values x̄ ± se
	1	2	3	4	Total	Wet weight x̄ ± se	Dry weight x̄ ± se	
372 ± 6	$\underline{15}$–2	$\underline{21}$–0	$\underline{19}$–0	$\underline{22}$–3	$\underline{77}$–5***	3.8 ± 0.3	0.9 ± 0.1	0.53 ± 0.02
176 ± 4	$\underline{11}$–0	$\underline{13}$–1	$\underline{30}$–2	$\underline{16}$–1	$\underline{70}$–4***	1.3 ± 0.3	0.32 ± 0.08	0.55 ± 0.04
93.96 ± 2.29	$\underline{41}$–1	$\underline{23}$–0	$\underline{7}$–0	$\underline{16}$–0	$\underline{87}$–1***	0.93 ± 0.12	0.20 ± 0.02	0.56 ± 0.02
46.71 ± 0.45	$\underline{6}$–2	$\underline{23}$–2	$\underline{27}$–0	$\underline{23}$–1	$\underline{79}$–5***	0.32 ± 0.05	0.07 ± 0.01	0.51 ± 0.01
18.44 ± 0.16	$\underline{17}$–3	$\underline{13}$–1	$\underline{7}$–0	$\underline{10}$–0	$\underline{47}$–4***	0.06 ± 0.02	0.01 ± 0.005	0.56 ± 0.01
7.20 ± 0.12	$\underline{4}$–4	$\underline{9}$–3	$\underline{8}$–1	$\underline{0}$–1	$\underline{21}$–9*	0.04 ± 0.01	0.009 ± 0.002	0.51 ± 0.01
2.61 ± 0.06	$\underline{0}$–0	$\underline{1}$–3	$\underline{1}$–1	$\underline{2}$–1	$\underline{4}$–5	0.015 ± 0.004	0.004 ± 0.001	0.58 ± 0.04

[a] g dry weight = 0.23 (g wet weight) + 0.07 (Product moment correlation coefficient = 0.99 ± 0.01 se; n = 25).

[b] The number of lobsters captured in abalone-baited traps is underlined.

[c] Differences between water flow index values are not significant (One-way ANOVA, with replicates, df = 6/21, F = 1.55, $P > 0.10$).

* The difference between the total number captured in abalone-baited *vs* unbaited traps is significant (Chi-square test for heterogeneity: df = 1, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

TABLE IV

Regression equations describing abalone effluence

Initial mass (g wet weight)	Equation[a]	Correlation coeff. (R ± se)	F test		
			d.f.	Value	*P*
372.	$A_t = A_0[1 - (0.01t + 0.05)]$	0.86 ± 0.06	1/12	34.20	<0.001
176.	$A_t = A_0[1 - (0.008t + 0.08)]$	0.84 ± 0.05	1/12	28.79	<0.001
93.96	$A_t = A_0[1 - (0.01t + 0.06)]$	0.91 ± 0.04	1/10	50.03	<0.001
46.71	$A_t = A_0[1 - (0.007t + 0.06)]$	0.87 ± 0.04	1/12	38.46	<0.001
18.44	$A_t = A_0[1 - (0.004t + 0.07)]$	0.54 ± 0.05	1/12	4.97	<0.05
7.20	$A_t = A_0[1 - (0.006t + 0.02)]$	0.64 ± 0.07	1/10	6.71	<0.05
2.61	$A_t = A_0[1 - (0.006t + 0.01)]$	0.65 ± 0.06	1/17	11.66	<0.005

[a] Predicted y-intercepts ($A_{t=0}$) closely approximate observed 1–8% mass losses incurred during transports of tissues to and from the field.

A_0: Initial mass before transport to the field; A_t: Final mass after transports and field exposures; t = 0: Field placement of abalone.

tissue using glass-distilled water, which we bound in polyacrylamide gel and used as bait. Control stimulants were identically prepared, except glass-distilled water was bound in gel without abalone muscle extract. Polyacrylamide gel is not susceptible to microbial decomposition and the small pore size prevents penetration by microbes (Allen *et al.*, 1975). Traps were paired at 8 stations with abalone gel (400 ml) placed

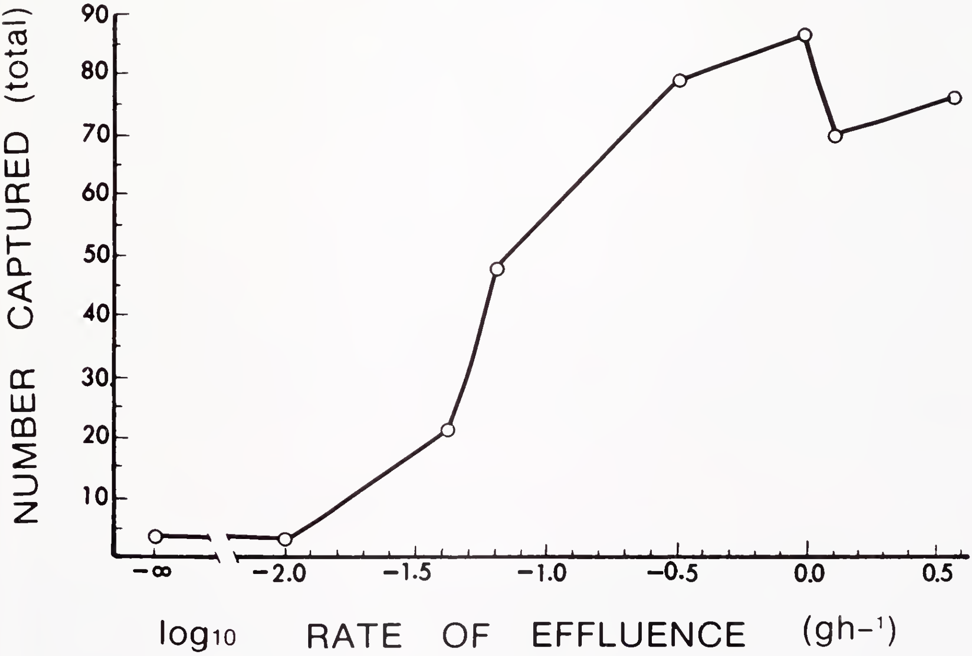

FIGURE 2. The log-linear relationship for captured lobsters, expressed as a function of the rate of abalone muscle effluence (wet weight). A log-linear dependence occurs over the −1.5 to −0.5 log unit interval. An effluence of negative infinity represents unbaited (control) traps, with the mean capture presented.

in one trap of each pair, while control gels were placed in alternative traps. Traps were raised each 24 h and gels were replaced and their positions reversed, for four consecutive days. Twenty-five lobsters were captured in traps having abalone gels, while only 6 lobsters were captured in traps having control gels, a significant difference (Chi-square test: $\chi^2 = 11.65$, d.f. = 1, $P < 0.001$). Abalone material lodged in gels was only 0.62 g/trap (dry weight) and the maximum amount of material released was 0.38 g trap^{-1} day^{-1}. This value compared favorably to the average total material released by 7.20 and 18.44 g abalone (0.22 and 0.33 g trap^{-1} day^{-1}, respectively). The number of lobsters captured in traps using abalone gels was also similar to the number captured in traps using 7.20 and 18.44 g abalone tissue (Table III), suggesting that the stimulatory capacity was nearly equal for effluence released by gels and by natural tissues. Microbial influences, therefore, appeared to be of minimal consequence in present experiments. Thresholds for locomotion (10^{-3} g/l) and for detection ($<10^{-7}$ g/l) were nearly identical in laboratory experiments using extract prepared from lyophilized tissues, as in experiments using AME (Zimmer-Faust *et al.*, unpub. data).

DISCUSSION

Trapping experiments were conducted in turbulent waters adjacent to breaking surf causing strong oscillatory on- and off-shore surges (20–50 cm/s) to be maintained and superimposed on weak longshore currents. Inman *et al.* (1968, 1971), investigating dispersions of dyes in similar surf zone waters, found that dye diffused by turbulences according to Fick's Law, primarily in on- and off-shore directions. Dye was also transported advectively by longshore currents, but longshore dilution was nearly zero for weak currents. Based on these findings, we apply Fick's Law to present data and estimate effective concentrations of abalone muscle effluence in waters adjacent to traps. In applying Fick's Law, we assume a weak longshore current, isotropic turbulence, and an ocean floor impervious to the flow of stimulants. For these conditions, the mean effluent concentration (N) at position (x, y, z) and time, $t \geq 0$ is:

$$(1) \qquad N(x, y, z, t) = \frac{q(t)}{4(\pi D)}\frac{3}{2}\int_0^t \exp\left\{\frac{[x - U(t - t')]^2 - y^2 - z^2}{4D(t - t')}\right\}\frac{dt'}{t - t'}$$

where q(t) is the rate of effluence, D is a diffusivity constant and U is the longshore current velocity. Abalone muscle was positioned at space coordinates, $x = y = z = 0$, and $t = 0$ was defined as the time of initial abalone placement. The rate of effluence was both continuous and constant, and depended slightly on the initial mass of abalone (Table IV). When time becomes large, effluence concentration (N) in equation 1 approaches the limit:

$$(2) \qquad N(x, y, z, t) = \frac{q}{2\pi Dr}\exp\left[\frac{-U}{2D}(r - x)\right]$$

where r is the vector distance from abalone $(x^2 + y^2 + z^2)^{1/2}$. It can be assumed that time is large in present experiments, because lobsters initiated foraging at dusk, 7–10 h (28,000–36,000 s) after bait placement of abalone (Lindberg, 1955; Winget, 1968; Carlberg, 1975; unpubl. obs.). Maximum concentrations occurred at positions lying on the x-axis, in line with the longshore current, where $r = x$ and $\exp[-U(r - x)(2D)^{-1}] = 1$. For present calculations, diffusivity constants (D) were taken as 0.03–5.9 m^2/s from Inman *et al.* (1971), and the minimum effective concentration was estimated by considering that 7.20 g abalone leached effluent at 0.04 g/h (10^{-5} g/s), yet attracted significant numbers of lobsters. For these conditions, effluent concentrations approached 3×10^{-7} to 2×10^{-9} g/l at trap perimeters, distances of only 17 cm from the abalone tissue source ($r = x = 0.17$ m). Concentrations become 6×10^{-8} to 4×10^{-10} g/l when converted to dry weight units. Asymptotic captures occurred when substances were leached at rates ≥ 0.32 g/h (Table III), producing concentrations $\geq 4 \times 10^{-7}$ to 5×10^{-9} g/l (dry weight). Diffusivity values were unlikely to cause error in present calculations, because values were taken for lower and higher turbulences than at our study site.

From the above calculations using natural tissues, two major findings appear: (1) Abalone muscle effluence evokes responses under field conditions at concentrations near laboratory-determined detection limits. *Panulirus* detected 10^{-8} to 10^{-10} g/l (dry weight) abalone muscle effluence (AME), as assayed by increased antennule flicking in laboratory experiments. Absolute thresholds for detection may be even lower. (2) Effective field concentrations of abalone muscle effluence were 4–6 log units more dilute than the laboratory-determined threshold for a chemical induction of locomotion. Lobsters initiated locomotion at concentrations $\geq 10^{-4}$ g/l AME, in laboratory tests. This high threshold for locomotion in *Panulirus* did not appear to be a laboratory artifact associated with tank construction, since other decapods tested in larger aquaria also initiate locomotion at similar concentrations, 2×10^{-6} to 3×10^{-5} g/l, when responding to various tissue extracts (Mackie and Shelton, 1972; Mackie, 1973; McLeese, 1973a; also see Pearson and Olla, 1977, for threshold estimates). This leads us to believe that chemical activation of distant foraging is unlikely in *Panulirus*. Chemical stimuli most likely initiate only local searches for food, where high stimulant concentrations are maintained. The insensitivity of the locomotor response and the preferential search with pereiopod dactyls were both adaptive for local but not for distant food searches.

Panulirus interruptus is known to locomote spontaneously at night (Lindberg, 1955; Winget, 1968; Krekorian *et al.*, 1974). Consequently, chemical induction of locomotion is unnecessary for distant foraging to occur. Based on our results, low concentrations appear to act by modulating, rather than by activating, distant locomotion patterns. This hypothesis is supported by our finding that baited traps captured lobsters nearly always at night, during the period of greatest endogenous activity (unpubl. data), whereas lobsters tested in the laboratory at night while inactive were found to be insensitive in their locomotor responses to chemical stimuli. It can always be argued that, in the laboratory, inability to dilute stimuli to effect lobster locomotor behavior results from laboratory-induced trauma. We admit this possibility, but feel the argument is unjustified since it can be applied indiscriminately to all laboratory investigations. Experiments are now needed to test responses of inactive and active animals held in large tanks simulating natural conditions. Even these experiments will not be without bias, however, since the natural flow dynamics of stimulants will not be reproduced.

We performed experiments using paired traps positioned 1–2 m apart, with bait tissues in one trap of each pair, leaving alternate traps empty. Initial bait placement was at random, and baits were replaced and their positions reversed from the first to the second day. Captures on consecutive days between paired traps with bait tissues of identical masses were compared, and in 4 out of the 8 trap stations, the number of lobsters captured in one trap was consistently greater than that of the alternate trap (Chi-square test: $P < 0.05$, 4 out of 8 comparisons). This means that the precise position of food is important in the attraction of lobsters and that at distances removed only 1–2 m from food, the influence of odor is greatly reduced.

Locomotion activated by stimuli not specifically related to a food source thus appears to be of vital importance, and the ability of lobsters to acquire food may depend on contacts with very limited chemically active spaces.

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D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

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THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except for a limited number of solicited review papers which may be accepted after formal refereeing), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

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Continued on Cover Three

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ORCHID FLORAL FRAGRANCES AND MALE EUGLOSSINE BEES: METHODS AND ADVANCES IN THE LAST SESQUIDECADE

NORRIS H. WILLIAMS[1] AND W. MARK WHITTEN[2]

[1]*Department of Natural Sciences, Florida State Museum, University of Florida, Gainesville, FL 32611, and* [2]*Department of Botany, University of Florida, Gainesville, FL 32611*

ABSTRACT

All species of the Neotropical subtribes Stanhopeinae and Catasetinae (Orchidaceae) are pollinated exclusively by male euglossine bees which are attracted to and collect the floral fragrances. The orchid-euglossine bee relationship is often highly specific: the flower of a given species of plant may attract males of only one or a few species out of dozens of euglossine species in the habitat. This pollinator specificity is based upon species-specific combinations of floral fragrance compounds which attract only one or a few species of euglossine bees. Such pollinator specificity is an important reproductive isolating mechanism between sympatric interfertile species of orchids. The male bees are thought to use the collected floral fragrance compounds in their own reproductive biology, probably as precursors of their own sex pheromones.

INTRODUCTION

One of the most striking examples of plant-insect interactions is that involving the male euglossine bees of the American tropics and the orchids they pollinate (Dodson, 1965). Although it was once thought that the male bees became "intoxicated" by the fragrances of the orchids, we now know that the situation is much different. In this paper we will review the progress that has been made since the 1969 paper on biologically active compounds in orchid floral fragrances (Dodson *et al.,* 1969). Some aspects of the biology of this group of insects were reviewed by Dressler (1982), and some aspects of the pollination biology of the orchids were reviewed by Williams (1982). Here we will emphasize the advances made in the collection and analysis of the floral fragrances, and the possible utilization of the floral fragrance compounds in the life of the insect.

Euglossine bees are exclusively Neotropical, and for the most part are solitary, communal, or quasisocial (depending on the particular species). There are three free-living genera: *Euglossa* (approximately 100 species, bright metallic blue, green, or bronze), *Eulaema* (13 species, brown or black, or striped hairy bees), and *Eufriesea* (52 species, metallic or brown/black and hairy). Two genera are nest parasites on the free-living groups: *Aglae* (monotypic, metallic blue) and *Exaerete* (5 species, metallic green). Taxonomic, biogeographic, and bibliographic references are given by Dressler (1979, 1982), Kimsey (1979, 1982), and Williams (1978, 1982).

The female bees gather food (pollen and nectar) from a variety of plants and they gather resins, mud, and other materials for nest building. The male bees visit some of the same plants as the females for food, but are not tied to the nest. The male bees leave the nest upon hatching and do not return to the nest again. They

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may live a vagabond life, or they may live in one general area for extended periods of time (Ackerman *et al.,* 1982).

Orchid flowers that exhibit the "male euglossine syndrome," or "euglossine pollination" do not provide food for the visiting insect; the pollen is hidden under the anther cap, and nectar is never produced. Only male bees are attracted to the flowers, and they are attracted solely by the floral fragrances. The bees enter the flower, brush at the area where the floral fragrance is produced (using specialized brushes on the front tarsi), launch into the air and transfer the collected floral fragrance to the inflated hind tibiae. The hind tibia of the male euglossine bee is inflated and contains specialized storage and glandular tissues (Cruz-Landim *et al.,* 1965).

The orchid flowers that these bees visit to collect fragrances have only one anther, which is hidden under the anther cap. The compacted pollen masses (pollinia) are attached to a stipe (derived from the epidermis of the stigma), which in turn is attached to a viscidium (also derived from a part of the stigma). The viscidium is very sticky and is the part of the entire unit (pollinarium) which becomes attached to the insect as it leaves the flower to launch into the air. Under proper conditions one or both of the pollinia may be deposited in the stigma as a bee carrying a pollinarium leaves the flower.

The members of the Orchidaceae that attract the male bees are also found exclusively in the Neotropics. Although the most interesting pollination mechanisms are found in the orchids, the bees also visit a number of species of other families to collect the floral fragrances: *Spathiphyllum* and *Anthurium* (Araceae), *Drymonia* and *Gloxinia* (Gesneriaceae), *Cyphomandra* (Solanaceae), and *Dalechampia* (Euphorbiaceae), all of which contain one or more species that attract the male bees (Williams and Dressler, 1976; Armbruster and Webster, 1979; Dressler, 1982; Williams, 1982).

All members of the subtribes Stanhopeinae and Catasetinae (and portions of several other subtribes; see Williams, 1982, for a review) are pollinated exclusively by male euglossine bees which are attracted to and collect the floral fragrances. The orchid-euglossine bee relationship is often highly specific; the flower of a given *Stanhopea* species (for example) may attract males of only one of a few species out of dozens of euglossine species in the habitat. This pollinator specificity is based upon species-specific combinations of floral fragrance compounds which attract only one or a few species of euglossine bees. Such pollinator specificity has been shown to be an important isolating mechanism in the genus *Catasetum* (Hills *et al.,* 1972). Also, Dodson (1970) blended cineole, benzyl acetate, and alpha-pinene to match the ratio found in the fragrance of *Stanhopea tricornis,* and found that the mixture attracted only two bee species. One was *Eulaema meriana,* the known pollinator of *S. tricornis;* the other was *Euglossa dodsoni,* a bee much too small to pollinate *S. tricornis.* Thus of a set of floral visitors, only a few species may have the appropriate size or behavior to pollinate the flower successfully. Selective attraction of different pollinators can thereby act as reproductive isolating mechanisms between otherwise interfertile species. The implications concerning sympatric speciation will be discussed later in this paper.

Early work on the euglossine syndrome by Vogel (1963a, b, 1966) and Dodson and his co-workers (Dodson *et al.,* 1969) led to several suggestions of why the male bees were collecting floral fragrances. Vogel suggested that perhaps the flowers were mimicking the appearance of the nests of the female bees, but Dodson *et al.* (1969) showed that this was not a viable suggestion. Dodson *et al.* offered three tentative hypotheses to explain why the male bees collect the floral fragrances. (1) The male bees use the floral fragrances as precursors of some compounds that they cannot normally manufacture, and thus extend their lives. This hypothesis was based on very limited data, and is now considered to be unattractive. (2) The male bees use the compounds unmodified to attract additional males of the same species to a mating site, or lek. Dodson (1975a) expanded on this hypothesis, but later studies by Kimsey (1980) do not support it. (3) Dodson *et al.* also suggested that the male bees might be using the floral fragrance compounds as precursors of a sex pheromone that would be used to attract females to a mating site. Although only a small amount of field work supports this hypothesis, it is now the favored one. In addition to being the hypothesis we favor most, it is also the one that is most complementary to the work that has been done on other groups of bees, most notably the work on bumblebees by Kullenberg and co-workers in Sweden (Kullenberg *et al.,* 1973).

Recent work on the collection of floral fragrances has centered on the use of adsorbents, although Holman has used oil impregnated glass fiber paper to collect floral fragrances. The first work reported by us on orchid floral fragrances involved the simple concentration of floral fragrances in plexiglas boxes and the direct injection of a 10 ml gas headspace sample into a gas chromatograph (Dodson and Hills, 1966; Hills *et al.,* 1968, 1972; Dodson *et al.,* 1969; Williams 1981, Williams *et al.,* 1981). This was an adequate method for the time, using ¼ inch packed metal columns in the gas chromatograph. We were able to identify tentatively a number of compounds from the floral fragrances of a variety of species of orchids by this method in conjunction with co-injections, comparing relative retention times, and simply smelling the peaks as they eluted from the end of the gas chromatograph column via an effluent splitter. However, this method did not allow one to obtain concentrated or liquid samples for additional chemical work, and as a result the progress on the identification of a number of the compounds in the floral fragrances came to a standstill. Bergstrom (1973) and his co-workers were apparently the first to use adsorbents to study floral fragrances. They re-worked the inlet system of their gas chromatograph to accept the pre-column collection tube, and the sample was directly injected onto the gas chromatograph column. Nilsson (1978) also used physical adsorbents to collect floral fragrances into a pre-column tube that was later directly inserted into the injection port of the gas chromatograph. The disadvantage of using a precolumn tube that is inserted directly into the injection port of the chromatograph is that all of the sample is used in one injection, and therefore the sample is not available for repeated injections. In addition, this requires a modification of the injection port of the gc which may not be feasible in some circumstances, such as when an instrument is used by a number of different investigators. An additional disadvantage is that the sample is usually destroyed, so that it is not possible to isolate individual (often unknown) compounds for additional chemical analyses. Holman (Holman and Heimermann, 1973) devised a technique using oil-impregnated glass fiber papers to collect floral fragrances. An advantage of his method is that the glass paper strips are easily mailed anywhere for field work, and no pumping mechanism is necessary for collecting the floral fragrances. There are, however, several disadvantages to his method. The method requires a reasonably elaborate preparation of the paper strips, it takes a long time to collect adequate amounts of the floral fragrance for analysis, and it was necessary to modify the injection port of the gas chromatograph.

Analytical Methods

We have recently developed a method that is a modification of the precolumn tube to use physical adsorbents, and devised a desorption device that allows us to collect a liquid sample of the floral fragrance. This method has several advantages:

(1) ease of sample preparation; (2) the production of a liquid sample that can be stored indefinitely; (3) production of an abundant sample so that part of the sample can be used for gc/ms analyses; (4) other parts can be used for preparatory gas chromatography to obtain pure samples of unknown compounds for NMR, IR, or other analytical techniques for structural determination. Furthermore, this method has the advantage that it does not require any modification of the injection port of the gas chromatograph.

The inflorescence is placed in a collecting chamber (plexiglas boxes, glass test tubes, or culture tubes, depending on the size and shape of the inflorescence or flower) and connected to a glass two-stage cartridge in an air stream. Fragrance laden air is drawn through the box and cartridge, with the air first coming into contact with the Tenax in the cartridge. The second stage of the cartridge is filled with charcoal to adsorb these compounds which are not adsorbed on the Tenax, or which were rapidly desorbed from the Tenax. Flow rate through the system is approximately 500 ml/minute, and sampling time is 3–4 hours.

Fragrance is desorbed from the cartridge by placing the cartridge in a desorbing device. This device was made from a length of copper tubing with reduction fittings on each end. A gas-tight seal is obtained by using a perforated high temperature septum at each end of the cartridge. The copper tube is heated to 200°C via the use of thermostated heating tape wrapped around the tube. One end of the tube is connected to a source of nitrogen gas with a flow rate of 30 ml/minute. The gas carrying the desorbed fragrance exits the device through a series of reduction fittings and flows through a 30 cm long glass capillary tube (1 mm diameter). The glass capillary tube fits inside a drilled aluminum block, which is itself fitted with a copper cold finger inserted into a Dewar flask filled with liquid nitrogen. There is therefore a temperature gradient established along the aluminum block and the fragrance compounds condense inside the glass capillary tube. After fifteen minutes of desorbing, the capillary tube is removed and the condensed compounds are eluted with one milliliter of pentane (or hexane, either of which is HPLC grade). The eluted sample and solvent is stored in a Teflon-capped automatic sampling vial for later analysis. This procedure yields sufficient fragrance for several hundred gc/ms analyses, and the samples can be stored indefinitely. The cartridges are easily made and are re-usable. The disadvantage of the system is that it requires a source of air flow, either a vacuum pump or a faucet aspirator, and thus is not an ideal system for field work. Additional details and schematics are given by Williams and Whitten (1982) and Williams (1983).

In order to discuss subtle qualitative and quantitative variations between plants, it is necessary to test the reproducibility of the sampling techniques. The variation in fragrance composition between the first and second day on anthesis of a *Catasetum viridiflavum* inflorescence is presented in Figure 1. Three replicate samples were taken each day (three adsorbent cartridges in parallel). The results indicate little variation between replicate samples and minor variation between days. Similar checks of variation between successively produced inflorescences reveal only minor quantitative differences. It is likely, however, that health of the plant and environmental conditions might affect fragrance compositions.

The floral fragrance samples are analyzed using gas chromatography/mass spectrometry. We currently use a Hewlett-Packard 5995B gc/ms system with electron impact ionization and fused silica capillary columns. Two 25 meter columns (OV-101) are inserted into the injection port. One column is routed to the mass spectrometer, and the second column is routed to a standard flame ionization detector (FID) and integrator. This arrangement allows us to obtain simultaneous mass spectra and integrated peak areas with a single injection.

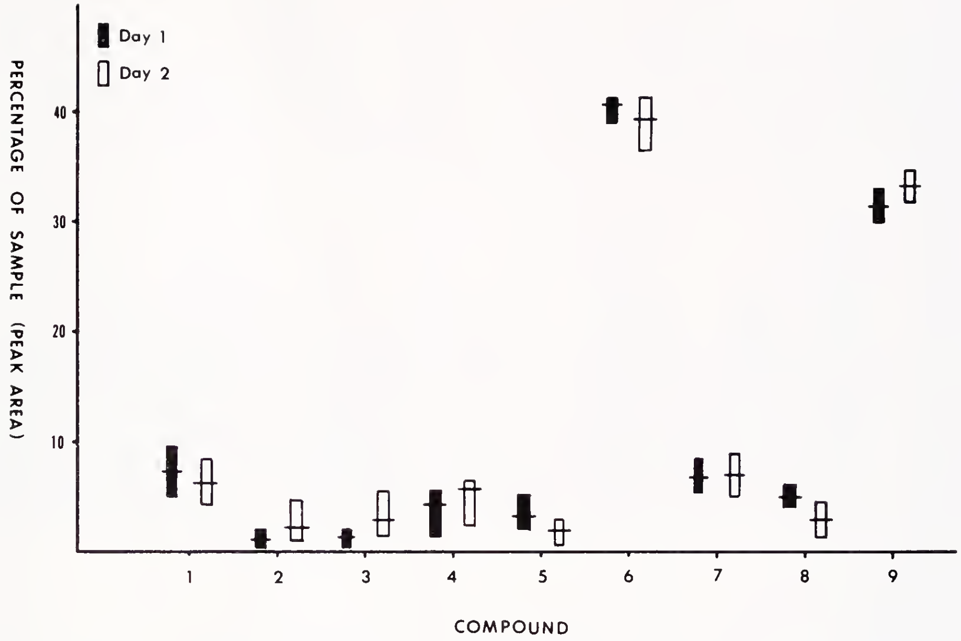


FIGURE 1. Variation in composition among replicated floral fragrance samples of a single inflorescence of *Catasetum viridiflavum*. The plant was sampled on the first and second day of anthesis, using three replicate samples (three adsorbent cartridges in parallel) per day. Bars denote the range in percent composition for each compound; horizontal marks indicate the mean.

Relevant gc/ms conditions are: helium carrier gas flow rate 1 ml/min; oven temperature programmed from 56°C to 280°C at 15°/min, T_1 = 2 min, T_2 = 26 min; wide bore 25 m fused silica OV-101 columns; spitless injection; injection port 300°C; transfer line 280°C; analyzer 180°C; source 150°C; FID 350°C; EM voltage 1400 V; open split interface between the column and source.

Unknown peaks of special interest can be isolated and purified via preparative gc using ¼ or ⅛ inch packed columns (OV-101 or Carbowax 20M) connected to an effluent splitter. The splitter diverts 90% of the eluting peak to an exit port where it can be collected (either with a chilled capillary or a short trap filled with Tenax). By trapping the fragrance of a number of inflorescences, it is possible to purify several milligrams of a given fragrance compound, which is sufficient for NMR, IR, and microchemical analyses and microchemical reactions (such as ozonolysis).

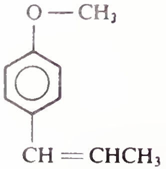
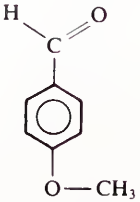
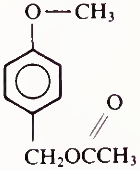
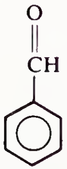
RESULTS

A number of chemicals attract male euglossine bees when presented in pure form in field trials. Many of these compounds also occur in orchid floral fragrances. Field tests of chemicals consist of simply tacking a 5 × 5 cm blotter pad to a tree or post in a forested habitat and saturating the pad with the compound to be tested. All bees attracted to the pad are collected for identification.

Table I contains those compounds identified in orchid floral fragrances, or compounds which are known to attract male euglossine bees. Most of the latter were discovered to be attractants by simply field testing large numbers of fragrant compounds. These two sets are not necessarily mutually inclusive for several reasons. First, the number of orchid species sampled is small, and new compounds will be

TABLE 1

Compounds presently known to attract male euglossine bees and/or which are known to occur in fragrances of orchids pollinated by male euglossine bees

Compound	Structure	Evidence[a]	Attractiveness[b] to male bees	Plant occurrence	Abundance[c]
anethole MW = 148.21			*	Not yet confirmed in fragrances.	
anisaldehyde MW = 136.15			*	Not yet confirmed in fragrances.	
anisyl acetate MW = 180.21 (p-methoxybenzyl acetate)		MS, RT-std	*	*Catasetum viridiflavum* *Gongora tricolor* *Spathiphyllum floribundum* (Araceae)	+ + ++
benzaldehyde MW = 106.12		MS, RT-std	*	*Aspasia principissa* *Catasetum viridiflavum* *Gongora bufonia* *Gongora truncata* *Spathiphyllum floribundum* *Stanhopea annulata* *Stanhopea impressa* *Stanhopea panamensis* *Stanhopea ruckeri* *Stanhopea wardii* *Trichopilia suavis*	+ + + + + + + + + + +

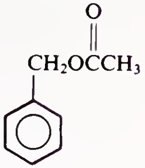
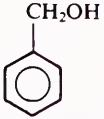
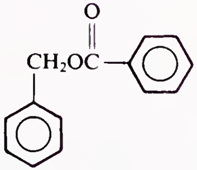
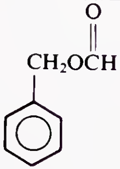
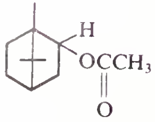
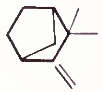
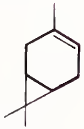
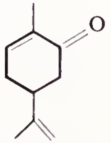
benzyl acetate MW = 150.12		MS, RT-std	**	*Aspasia principissa*	+
				Aspasia variegata	+
				Catasetum expansum	++
				Catasetum longifolium	++
				Catasetum viridiflavum	++
				Clowesia thylaciochila	+
				Gongora quinquenervis	++
				Spathiphyllum floribundum	+++
				Stanhopea annulata	++
				Stanhopea costaricensis	+
				Stanhopea impressa	+
				Stanhopea panamensis	+
				Stanhopea pulla	++,+++
				Stanhopea ruckeri	+
				Stanhopea tigrina	+
				Stanhopea wardii	+
benzyl alcohol MW = 108.14		MS, RT-std	*	*Catasetum expansum*	++
				Catasetum viridiflavum	+
				Stanhopea annulata	+
				Stanhopea impressa	++
benzyl benzoate MW = 212.25		MS, RT-std	*	*Aspasia principissa*	+
				Catasetum expansum	+
				Catasetum longifolium	++
				Catasetum viridiflavum	+
				Clowesia russelliana	+
				Cycnoches sp.	++
				Gongora cassidea	+
				Gongora quinquenervis	+
				Stanhopea panamensis	+,++,+++
				Stanhopea pulla	+
benzyl formate MW = 136.15			*	Not yet confirmed in fragrances.	

TABLE I (*Continued*)

Compound	Structure	Evidence[a]	Attractiveness[b] to male bees	Plant occurrence	Abundance[c]
bornyl acetate MW = 196.29			*	Not yet confirmed in fragrances.	
camphene MW = 136.24		MS, RT-std	—	*Aspasia variegata*	+
				Catasetum expansum	+
				Catasetum macroglossum	+
				Catasetum maculatum	+
				Catasetum viridiflavum	+
				Clowesia russelliana	+
				Gongora cassidea	+
				Gongora quinquenervis	+,++
				Stanhopea costaricensis	+
				Stanhopea ecornuta	+
				Stanhopea gibbosa	+
				Stanhopea pulla	+
				Stanhopea wardii	+
				Vanilla pompona	+
Δ-3-carene MW = 136.24		MS, RT-std	—?	*Mormodes hookeri*	+
carvone MW = 150.22		MS, RT-std	*	*Catasetum expansum*	++
				Catasetum longifolium	++
				Catasetum viridiflavum	+,++

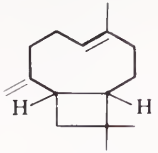
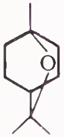
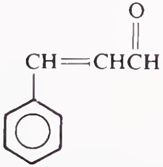
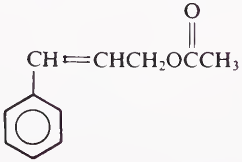
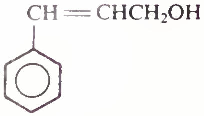
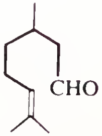
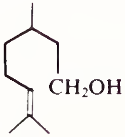
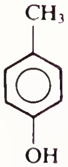
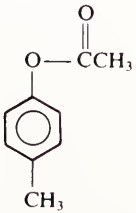
β-caryophyllene MW = 204.36		MS, RT-std	—	*Gongora armeniaca*	++
				Gongora quinquenervis	+
				Gongora tricolor	++
1,8-cineole MW = 154.25		MS, RT-std	**	*Aspasia variegata*	++
				Clowesia russelliana	+
				Embreea rodigasiana	+
				Gongora aceras	++
				Gongora atropurpurea	+
				Gongora cassidea	+
				Gongora gibba	++
				Gongora quinquenervis	++
				Stanhopea annulata	+
				Stanhopea candida	+
				Stanhopea costaricensis	++,+++
				Stanhopea ecornuta	+++
				Stanhopea gibbosa	+++
				Stanhopea impressa	++
				Stanhopea oculata	++
				Stanhopea pulla	++
				Stanhopea ruckeri	++
				Stanhopea wardii	+,++
				Vanilla pompona	++
cinnamic aldehyde MW = 132.16	 $C_6H_5CH{=}CHCH{=}O$	MS, RT-std	*	*Clowesia thylaciochila*	+
cinnamyl acetate MW = 176.22	 $C_6H_5CH{=}CHCH_2OC({=}O)CH_3$	MS, RT-std	*	*Clowesia thylaciochila*	++ (trans)
				Stanhopea tigrina	+ (cis) ++ (trans)
				Zygopetalum mackayi	+ (trans)

TABLE I (*Continued*)

Compound	Structure	Evidence[a]	Attractiveness[b] to male bees	Plant occurrence	Abundance[c]
cinnamyl alcohol MW = 134.18		MS, RT-std	*	*Clowesia thylaciochila*	+
citronellal MW = 154.25		MS, RT-std	*	Not known in species that are pollinated by male bees, but is found in species of *Brassavola*.	
citronellol MW = 156.27		MS, RT-std	*	Not known in species that are pollinated by male bees, but is found in species of *Brassavola*.	
p-cresol MW = 108.14		MS, RT-std	**	*Gongora tricolor*	++
p-cresyl acetate MW = 150.18			**	Not yet confirmed in fragrances.	

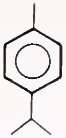
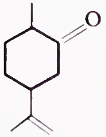
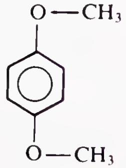
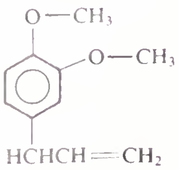
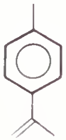
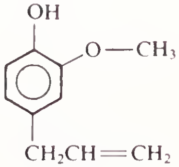
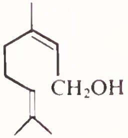
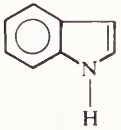
p-cymene MW = 134.22		MS, RT-std	—	*Aspasia variegata*	++
				Catasetum expansum	++
				Catasetum integerrimum	+
				Catasetum longifolium	+
				Catasetum macroglossum	++
				Catasetum maculatum	+
				Catasetum viridiflavum	+
				Clowesia russelliana	+
				Clowesia warczewitzii	+
				Gongora aceras	+
				Gongora bufonia	++
				Gongora cassidea	+
				Gongora gibba	+
				Gongora quinquenervis	+,++
				Gongora tricolor	+
				Gongora truncata	+
				Gongora unicolor	++
				Mormodes sinuatum	+
				Stanhopea candida	+
				Stanhopea costaricensis	0,+++
				Stanhopea ecornuta	+
				Stanhopea impressa	+
				Stanhopea aff. *impressa*	+++
				Stanhopea oculata	+++
				Stanhopea panamensis	+
				Stanhopea pulla	+,+++
				Stanhopea ruckeri	+++
				Stanhopea tigrina	+
				Stanhopea wardii	+,++
dihydrocarvone MW = 152.24		MS, RT-std	?	*Catasetum expansum*	+
				Catasetum viridiflavum	++
p-dimethoxy benzene MW = 138.16		MS, RT-std	**	*Cycnoches loddigesii*	++
				Embreea rodigasiana	+
				Gongora cassidea	+
				Gongora truncata	++
				Mormodes hookeri	+++
				Mormodes sinuatum	++

TABLE I (*Continued*)

Compound	Structure	Evidence[a]	Attractiveness[b] to male bees	Plant occurrence	Abundan
1,2-dimethoxy-4(2-propenyl) benzene MW = 178.33		MS-lib	?	*Zygopetalum mackayi*	+
α,p-dimethyl styrene MW = 132.21		MS, RT-std	?	*Catasetum expansum* *Catasetum integerrimum* *Catasetum macroglossum* *Catasetum viridiflavum* *Clowesia warczewitzii* *Gongora tricolor*	+ + + + + +
eugenol MW = 164.21		MS, RT-std	**	*Gongora aceras* *Gongora atropurpurea* *Gongora quinquenervis* *Spathiphyllum floribundum*	++ +++ + +
geraniol MW = 154.26		MS, RT-std	*	Not known in species that are pollinated by male bees, but is found in species of *Brassavola*.	
indole MW = 117.15		MS, RT-std	*	*Cycnoches loddigesii* *Gongora cassidea* *Gongora quinquenervis* *Gongora tricolor* *Stanhopea candida* *Stanhopea* aff. *impressa* *Stanhopea tigrina*	+ + + + ++ ++ +

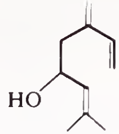
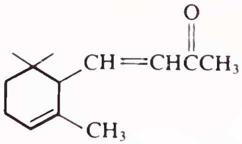
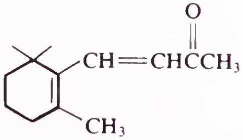
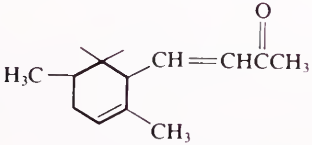
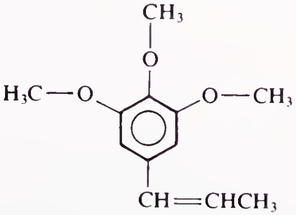
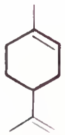
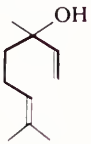
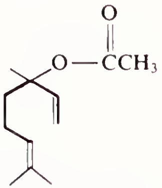
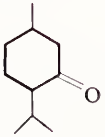
ipsdienol MW = 152.24		MS-lib	?	*Clowesia warczewitzii*	++
α-ionone MW = 192.30			*	Not yet confirmed in fragrances.	
β-ionone MW = 192.30			*	Not yet confirmed in fragrances.	
α-irone MW = 206.33			*	Not yet confirmed in fragrances.	
isoelemicin MW = 208.26		MS-lib	?	*Clowesia warczewitzii* *Gongora gibba* *Gongora tricolor*	+ +++ +

TABLE I (*Continued*)

Compound	Structure	Evidence[a]	Attractiveness[b] to male bees	Plant occurrence	Abundance[c]
limonene MW = 136.24		MS, RT-std	*	*Catasetum expansum* *Catasetum integerrimum* *Catasetum longifolium* *Catasetum macroglossum* *Catasetum maculatum* *Catasetum viridiflavum* *Gongora aceras* *Gongora quinquenervis* *Stanhopea panamensis* *Vanilla pompona*	+ ++ + + + + + +,++ + +
linalool MW = 154.25		MS, RT-std	*	*Gongora bufonia* *Gongora cassidea*	++ +
linalyl acetate MW = 196.26			*	Not yet confirmed in fragrances.	
dl-menthone MW = 154.25			*	Not yet confirmed in fragrances.	

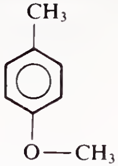
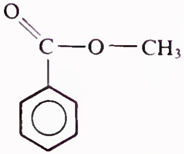
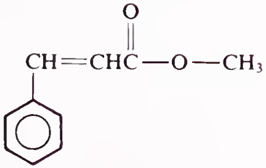
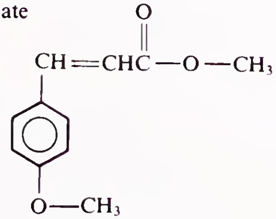
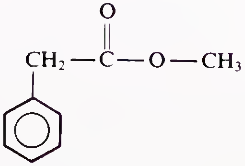
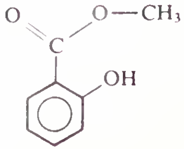
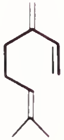
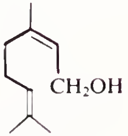
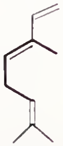
p-methyl anisole MW = 122.17		MS, RT-std	?	*Gongora tricolor*	++
methyl benzoate MW = 136.15		MS, RT-std	**	*Catasetum maculatum* *Cycnoches* sp. *Stanhopea candida* *Stanhopea panamensis*	+ + + +,++
methyl cinnamate MW = 162.19		MS, RT-std	**	*Aspasia epidendroides* *Embreea rodigasiana* *Gongora cassidea* *Gongora quinquenervis* *Gongora superflua* *Gongora truncata* *Stanhopea candida* *Stanhopea costaricensis* *Stanhopea ecornuta* *Stanhopea embreei*	+,+++ + + + ++ + + + + +++
methyl-p-methoxy cinnamate MW = 192.21		MS, RT-std	*	*Gongora quinquenervis*	++
methyl phenyl acetate MW = 150.18		MS, RT-std	*	*Gongora quinquenervis*	+

TABLE I (*Continued*)

Compound	Structure	Evidence[a]	Attractiveness[b] to male bees	Plant occurrence	Abundance[c]
methyl salicylate MW = 152.14		MS, RT-std	**	*Cycnoches* sp.	++
				Stanhopea candida	+++
				Stanhopea panamensis	0,+,++,+++
myrcene MW = 136.24		MS, RT-std	*	*Catasetum viridiflavum*	+
				Cycnoches sp.	+
				Gongora bufonia	+
				Gongora gibba	+
				Gongora quinquenervis	+,++
				Gongora tricolor	+
				Stanhopea annulata	+,++
				Stanhopea costaricensis	++
				Stanhopea ecornuta	++
				Stanhopea gibbosa	++
				Stanhopea impressa	++
				Stanhopea aff. *impressa*	++
				Stanhopea oculata	++
				Stanhopea panamensis	+
				Stanhopea ruckeri	++
				Stanhopea wardii	+
nerol MW = 154.25		MS, RT-std	*	*Clowesia warczewitzii*	++
β-ocimene MW = 136.24		MS, RT-std	*	*Aspasia principissa*	++
				Clowesia warczewitzii	+
				Gongora armeniaca	++
				Gongora gibba	+
				Gongora quinquenervis	+,++
				Vanilla pompona	+

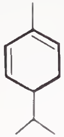
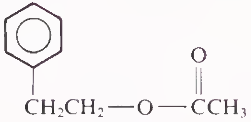
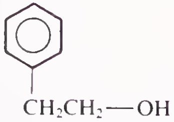
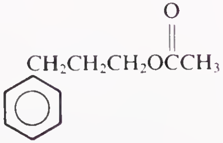
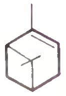
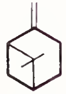
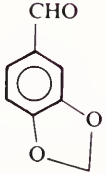
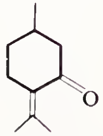
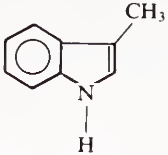
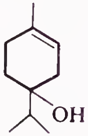
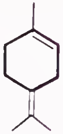
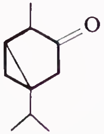
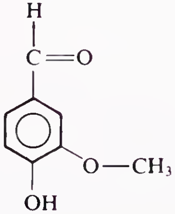
α-phellandrene MW = 136.24		MS, RT-std	*	*Catasetum expansum*	+
				Catasetum viridiflavum	+
2-phenylethyl acetate MW = 164.21		MS, RT-std	**	*Catasetum viridiflavum*	++
				Clowesia russelliana	++
				Gongora aceras	+
				Gongora armeniaca	+
				Gongora bufonia	++
				Gongora tricolor	++
				Gongora truncata	++
				Stanhopea annulata	++
				Stanhopea costaricensis	+,++
				Stanhopea impressa	++
				Stanhopea oculata	+
				Stanhopea panamensis	+
				Stanhopea tigrina	+++
				Stanhopea wardii	+++
				Trichopilia suavis	+
2-phenylethyl alcohol MW = 122.17		MS, RT-std	**	*Catasetum viridiflavum*	+
				Clowesia russelliana	+
				Gongora aceras	++
				Gongora bufonia	+
				Gongora quinquenervis	+
				Gongora tricolor	++
				Stanhopea annulata	+
				Stanhopea costaricensis	0,+,++
				Stanhopea impressa	+
				Stanhopea tigrina	+
				Stanhopea wardii	++
phenylpropyl acetate MW = 178.23		MS-lib	?	*Clowesia thylaciochila*	+

TABLE I (*Continued*)

Compound	Structure	Evidence[a]	Attractiveness[b] to male bees	Plant occurrence	Abundance[c]
α-pinene MW = 136.24		MS, RT-std	** [only (−) isomer is an attractant]	*Aspasia variegata* *Catasetum expansum* *Catasetum integerrimum* *Catasetum longifolium* *Catasetum macroglossum* *Catasetum maculatum* *Catasetum viridiflavum* *Clowesia russelliana* *Gongora aceras* *Gongora quinquenervis* *Stanhopea costaricensis* *Stanhopea ecornuta* *Stanhopea gibbosa* *Stanhopea pulla* *Stanhopea wardii*	+ + + + + + ++ + + +,++ + + + + +
β-pinene MW = 136.24		MS, RT-std	—	*Catasetum viridiflavum* *Gongora quinquenervis*	+ +
piperonal MW = 150.13			*	Not yet confirmed in fragrances.	
pulegone MW = 152.24			*	Not yet confirmed in fragrances.	

skatole MW = 131.17 [3-methyl indole]			**	Not yet confirmed in fragrances.	
terpinen-4-ol MW = 154.25		MS, RT-std	**	*Gongora gibba* *Gongora quinquenervis*	++ ++,+++
terpinolene MW = 136.24			?	*Gongora quinquenervis*	+
thujone MW = 152.24			*	Not yet confirmed in fragrances.	
vanillin MW = 152.14			**	Vanillin is not known to occur in the fragrance of any orchid, although it does occur in the ripe fruits of species of *Vanilla*.	

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[a] Evidence: MS, Rt-std = match of mass spectrum and retention time with those of known standard. MS lib = match with mass spectral library only.

[b] Attractiveness: * = moderate attractant; ** = good attractant.

[c] Abundance: + = minor component (0–20%); ++ = major component (21–90%); +++ = >90% of fragrance. Multiple symbols indicate intraspecific variation in fragrance composition.

added as more taxa are sampled. Also, there are many compounds in the sampled fragrances which we have not yet been able to identify and have not included in the table. Most of these appear to be sesquiterpenes and their derivatives. The number of possible isomers is large, and the minute amount of sample makes identification difficult. Second, several of the known fragrance components do not attract male bees when presented in pure form in field trials. Examples of these include p-cymene and camphene. These compounds are common in orchid fragrances, but do not attract any bees; instead they appear to modify the attractiveness of other compounds, such as cineole, resulting in the selective attraction of fewer species of bees. Some compounds (*e.g.* vanillin, skatole, p-cresyl acetate) are good "baits" for male bees, but have not been found in fragrances. Further sampling may reveal such compounds in fragrances. Alternatively, they may represent analogs of naturally occurring fragrance compounds and attract bees due to their similar structure. Examples are indole and p-cresol, and their respective analogs, skatole and p-cresyl acetate.

The data on attractiveness of the compounds to male bees should be regarded with some caution. This information has been accumulated over a 15-year period of baiting for bees, primarily from unpublished data of Dodson, Dressler, and ourselves and co-workers. Some of the compounds listed as poor attractants have been tested only a few times, and the chemical purity and isomeric composition of some baits was unknown. Also, many of the compounds are chiral and exist as two or more enantiomers. We have no information regarding the stereochemistry of the chiral fragrance compounds as they occur in orchids, but such information may prove to be important. We have recently baited with (+) and (−) isomers of several compounds (limonene, carvone, and alpha-pinene). In many previous tests dl-alpha-pinene has never attracted any bees. To our surprise, (−) alpha-pinene proved to be a good attractant of *Eulaema nigrita,* whereas (+) alpha-pinene and a racemic mixture attracted no bees. It appears that at least some species of euglossine bees can discriminate between stereoisomers, and a complete characterization of an orchid fragrance would ideally include the stereochemical configuration of the chiral components. Optically-active chromatographic packings have recently been introduced which allow enantiomers to be resolved, and identified by co-chromatography with known standards. The use of such packings should allow more complete characterizations of fragrances.

A number of the compounds in the table were only recently identified, and we have not yet had the opportunity to test them extensively in field trials. We suspect that many of them will prove to be good attractants, especially p-dimethoxy benzene, isoelemicin, methyl-p-methoxycinnamate, and terpinen-4-ol, since these compounds are the major components of various orchid fragrances.

Species specificity, variations, and distribution of floral fragrances

We tentatively identified a number of floral fragrances and discussed their distribution in the genus *Catasetum* (subtribe Catasetinae) a decade ago (Hills *et al.,* 1972). We have also given the tentative identification and distribution of additional floral fragrance compounds in the genera *Anguloa, Lycaste, Mendoncella, Acineta, Houlettia, Luddemannia, Lycomormium, Paphinia,* and *Sievekingia* (Williams, Atwood, and Dodson, 1981; Williams, Whitten, and Dodson, 1983). All of this work was based on the headspace sampling technique developed at the University of Miami in the late 1960's and early 1970's. In this paper we will report on the confirmation of many of those identifications by mass spectrometry and the identification of additional compounds in some genera. We will use *Stanhopea* (a genus of about 50 species occurring throughout much of the Neotropics from northwestern Mexico to southeastern Brazil) as a detailed example of the variation and species-specificity in floral fragrance composition, and we will attempt to correlate variation in pollinators with differences in floral fragrance. Detailed information will also be given for a part of the genus *Catasetum.*

Unlike many other chemotaxonomic characters, the adaptive value of floral fragrances to male euglossine bee pollinated orchids is known; furthermore, the effects of variation in fragrance composition can (in theory) be related to differences in the pollinator sets attracted to the different fragrance forms. Floral fragrance composition should be useful in delimiting reproductively isolated groups within these taxa. Previous studies of *Stanhopea* floral fragrance (Dodson *et al.,* 1969; Dodson and Hills, 1966; Hills *et al.,* 1968) utilized direct injection of headspace samples into a gas chromatograph. These studies were successful in identifying some of the major fragrance compounds, and also demonstrated considerable interspecific variation in fragrances. In this study we have used combined gas chromatography/mass spectrometry to study variation in floral fragrance composition among 33 plants of 14 *Stanhopea* species and one species of *Embreea* previously included in *Stanhopea.*

In most *Stanhopea* species all flowers of a given inflorescence open simultaneously and persist for only two to five days before wilting. Fragrance production is strongest during the morning (about 0800 to 1300) which corresponds to the period of greatest fragrance collecting activity of the male bees. Fragrance production essentially ceases at night. All fragrance samples were collected between 0800 and 1300 hours on the first day of anthesis of each plant.

The plants used in this study were obtained from the Marie Selby Botanical Gardens (Sarasota, FL) and from the University of Florida. Collection localities and greenhouse accession numbers are given in Table II. Liquid preserved vouchers are deposited in our collection at the University of Florida and herbarium vouchers will be deposited at SEL. Plants were cultivated under uniform (as possible) greenhouse conditions for at least one year prior to sampling. Sampling techniques were described above.

The floral fragrance composition of the 33 plants are presented in Table III. Compounds comprising less than 1% of the total fragrance were not included in the table. A total of 50 compounds (>1%) was detected in the samples of 15 species. Eighteen of the compounds, including most of the major constituents, were identified on the basis of mass spectra and retention times. The data in Table III can be summarized as follows:

1. *Stanhopea panamensis* is characterized by large percentages of benzyl benzoate and methyl salicylate and/or methyl benzoate.
2. *Stanhopea wardii* is characterized by large percentages of phenylethyl acetate, phenylethyl alcohol, p-cymene, and cineole, but is quantitatively variable. One plant (#11) produces large amounts of benzyl benzoate.
3. *Stanhopea embreei* is unique in producing only methyl cinnamate.
4. *Stanhopea ruckeri* fragrance is distinctive; it is composed of benzyl benzoate, p-cymene, cineole, and myrcene. The presence of cineole and the lack of methyl salicylate distinguish it from *S. panamensis;* the absence of phenylethyl acetate distinguishes it from *S. wardii.*
5. The three samples of *Stanhopea costaricensis* differ qualitatively. Plant #16 is dominated by p-cymene, phenylethyl alcohol, and cineole; plant #17 is dominated

TABLE 11

Collection localities and greenhouse accession numbers of Stanhopea *plants used in this study*

#	Species	Locality	Accession #[a]
1	*Stanhopea panamensis* Dodson ined.	Cerro Campana, Panama, Panama	UF-93
2	*Stanhopea panamensis* Dodson ined.	Cerro Campana, Panama, Panama	UF-25
3	*Stanhopea panamensis* Dodson ined.	unknown	UF-29
4	*Stanhopea panamensis* Dodson ined.	Cerro Campana, Panama, Panama	UF-35
5	*Stanhopea panamensis* Dodson ined.	unknown	UF-47
6	*Stanhopea panamensis* Dodson ined.	Cerro Campana, Panama, Panama	UF-80
7	*Stanhopea panamensis* Dodson ined.	Cerro Campana, Panama, Panama	UF-69
8	*Stanhopea panamensis* Dodson ined.	unknown	UF-91
9	*Stanhopea wardii* Lodd. ex Lindl.	Nicaragua	UF-26
10	*Stanhopea wardii* Lodd. ex Lindl.	Rio Chiriqui, Chiriqui, Panama	UF-39
11	*Stanhopea wardii* Lodd. ex Lindl.	Santa Clara, Chiriqui, Panama	UF-41
12	*Stanhopea wardii* Lodd. ex Lindl.	Pinola, Chiriqui, Panama	UF-58
13	*Stanhopea wardii* Lodd. ex Lindl.	unknown	SEL 48-465
14	*Stanhopea wardii* Lodd. ex Lindl.	Panama	SEL 23-75-31
15	*Stanhopea oculata* (Lodd.) Lindl.	Nicaragua	UF-48
16	*Stanhopea costaricensis* Rchb. f.	Nicaragua	UF-13
17	*Stanhopea costaricensis* Rchb. f.	Cerro Campana, Panama, Panama	UF-43
18	*Stanhopea costaricensis* Rchb. f.	Nicaragua	UF-59
19	*Stanhopea gibbosa* Rchb. f.	Nicaragua	UF-33
20	*Stanhopea embreei* Dodson	Ecuador	UF-213
21	*Stanhopea ruckeri* Lindl.	Nicaragua	UF-42
22	*Stanhopea ruckeri* Lindl.	Bocaycito, Nicaragua	UF-55
23	*Stanhopea impressa* Rolfe	Santo Domingo, Las Palmas, Ecuador	UF-36
24	*Stanhopea* aff. *impressa*	Piñas, El Oro, Ecuador	UF-40
25	*Stanhopea tigrina* Batem. ex Lindl.	unknown	SEL 23-75-41
26	*Stanhopea ecornuta* Lem.	unknown	UF-60
27	*Stanhopea pulla* Rchb. f.	Rio Iguanita, Colon, Panama	UF-65
28	*Stanhopea pulla* Rchb. f.	Rio Iguanita, Colon, Panama	UF-66
29	*Stanhopea annulata* Mansf.	Rio Chiquilpe, Ecuador	SEL 46-75-60
30	*Stanhopea annulata* Mansf.	Rio Palenque, Los Rios, Ecuador	UF-53
31	*Stanhopea anfracta* Rolfe	Moyabamba, Peru	SEL-23-75-38#2
32	*Stanhopea candida* Barb. Rodr.	unknown	SEL 79-1490
33	*Embreea rodigasiana* (Claes. ex Cogn.) Dodson (=*Stanhopea rodigasiana* Claes. ex Cogn.)	unknown	UF-94

[a] UF = University of Florida orchid collection (N. H. Williams); SEL = The Marie Selby Botanical Gardens, Sarasota, Florida.

by cineole and myrcene, and plant #18 produces cineole, phenylethyl alcohol, and myrcene.

6. *Stanhopea gibbosa* is dominated by cineole and myrcene, and resembles plant #17 of *S. costaricensis.*

7. *Stanhopea tigrina* is distinguished by a large percentage of phenylethyl acetate and the presence of cinnamyl acetate and indole.

8. *Stanhopea oculata* is dominated by cineole, p-cymene, and myrcene.

9. The two samples of *Stanhopea pulla* are qualitatively similar to each other, but differ in the relative amounts of p-cymene and benzyl acetate.

10. *Stanhopea annulata* is distinguished by large percentages of phenylethyl acetate, benzyl acetate, phenylethyl alcohol, and an unidentified compound (rt = 7.40).

TABLE III

Floral fragrance composition of Stanhopea *samples*

Compound and retention time		1. *S. panamensis*	2. *S. panamensis*	3. *S. panamensis*	4. *S. panamensis*	5. *S. panamensis*	6. *S. panamensis*	7. *S. panamensis*	8. *S. panamensis*	9. *S. wardii*
4.60		—	—	—	—	—	—	—	—	—
4.75	benzaldehyde	3.6	11.2	3.4	6.6	1.6	4.5	3.1	26.0	1.2
4.77	α-pinene	—	—	—	—	—	—	—	—	—
4.79		—	—	—	—	—	—	—	—	—
4.96	camphene	—	—	—	—	—	—	—	—	—
4.99		—	—	—	—	—	—	—	—	—
5.01		1.0	—	—	—	—	—	—	—	—
5.51	myrcene	—	—	—	—	—	—	—	1.0	—
5.93	p-cymene	5.0	—	1.6	—	2.2	7.2	—	2.3	13.9
5.97		—	4.7	—	—	—	—	—	—	—
6.04	limonene	1.0	—	—	—	—	—	1.0	—	—
6.05	1,8 cineole	—	—	—	—	—	—	—	—	5.6
6.07	benzyl alcohol	—	—	—	—	—	—	—	—	—
6.25		—	—	—	—	—	—	—	—	—
6.27		—	—	—	—	—	—	—	—	—
6.68	methyl benzoate	8.4	3.0	4.9	1.5	—	14.7	3.5	24.7	—
6.73		—	—	—	—	—	—	—	—	—
6.74		—	—	—	—	—	—	—	—	—
6.75		—	—	—	—	—	—	—	—	—
6.78		—	—	—	—	—	—	—	—	—
6.79		—	—	—	—	—	—	—	—	—
6.84		—	—	—	—	—	1.0	—	—	—
6.89		—	1.7	—	—	—	—	—	—	—
6.95	2-phenylethyl alcohol	—	—	—	—	—	—	—	—	27.2
6.97		—	—	—	—	1.0	—	—	—	—
7.33		—	—	—	—	—	—	—	—	—
7.40		—	—	—	—	—	—	—	—	—
7.44	benzyl acetate	4.5	—	—	—	—	—	—	—	—
7.49	p-dimethoxybenzene	—	—	—	—	—	—	—	—	—
7.89		—	—	—	—	—	1.5	—	—	—
7.91		—	—	—	—	—	—	—	—	—
7.94	methyl salicylate	—	40.4	2.3	64.7	39.7	—	84.9	1.5	—
8.00		3.6	—	—	—	—	—	—	—	—
8.65	2-phenylethyl acetate	4.6	—	—	—	—	—	—	—	47.1
8.73		3.1	—	—	—	—	—	—	—	—
9.00	indole	—	—	—	—	—	—	—	—	—
9.69	cinnamyl acetate (I)	—	—	—	—	—	—	—	—	—
9.85	methyl cinnamate	—	—	—	—	—	—	—	—	—
10.45	cinnamyl acetate (II)	—	—	—	—	—	—	—	—	—
11.14		—	—	—	—	—	—	—	—	—
11.73		—	—	—	—	—	—	—	—	—
12.14		—	—	—	—	—	—	—	—	—
13.82	benzyl benzoate	59.2	37.4	87.0	23.5	53.5	59.4	5.9	44.2	—
14.04		—	—	—	—	—	—	—	—	—

11. *Stanhopea anfracta* is dominated by myrcene and an unidentified compound (rt = 7.40) also present in *S. annulata.*

12. *Stanhopea candida* is distinguished by the presence of methyl salicylate and indole.

TABLE III (*Continued*)

	10. *S. wardii*	11. *S. wardii*	12. *S. wardii*	13. *S. wardii*	14. *S. wardii*	15. *S. oculata*	16. *S. costaricensis*	17. *S. costaricensis*	18. *S. costaricensis*	19. *S. gibbosa*	20. *S. embreei*	21. *S. ruckeri*	22. *S. ruckeri*
4.60	—	—	—	—	—	—	—	—	—	—	—	—	—
4.75	1.0	2.5	—	1.0	—	—	—	—	—	—	—	2.6	2.5
4.77	—	—	1.7	1.0	—	—	—	1.5	1.8	1.8	—	—	—
4.79	—	—	—	—	—	—	—	—	—	—	—	—	—
4.96	—	—	1.6	—	—	—	1.0	—	1.1	1.3	—	—	—
4.99	—	—	—	—	—	2.8	—	—	—	—	—	—	—
5.01	—	—	—	—	—	—	—	1.0	—	—	—	—	—
5.51	—	—	1.4	—	—	9.1	1.9	13.3	6.8	20.6	—	8.4	2.0
5.93	2.8	2.2	3.1	3.5	1.0	29.2	42.4	—	1.7	—	—	10.5	41.8
5.97	—	—	—	—	—	—	—	—	—	—	—	—	—
6.04	—	—	—	—	—	—	—	—	—	—	—	—	—
6.05	4.2	1.0	13.7	2.0	8.8	30.1	7.7	68.3	55.2	71.4	—	19.5	19.5
6.07	—	—	—	—	—	—	—	—	—	—	—	—	—
6.25	—	—	—	—	—	—	—	—	—	—	—	—	—
6.27	—	—	—	—	—	—	—	—	—	2.2	—	—	—
6.68	—	—	—	—	—	—	—	—	—	—	—	2.9	—
6.73	—	—	—	—	—	—	—	—	—	—	—	—	—
6.74	—	—	—	—	—	—	—	—	—	—	—	—	—
6.75	—	—	—	—	—	—	4.9	—	—	—	—	—	—
6.78	—	—	—	—	—	3.8	—	—	—	—	—	—	—
6.79	—	—	—	—	—	—	—	1.0	—	—	—	—	—
6.84	—	—	—	—	—	—	—	—	—	—	—	—	—
6.89	—	—	—	—	—	—	—	—	—	—	—	—	—
6.95	17.4	8.7	13.1	7.4	6.2	—	31.5	—	1.9	—	—	—	—
6.97	—	—	—	—	—	—	—	—	—	—	—	—	—
7.33	—	—	—	—	—	—	—	—	—	—	—	—	—
7.40	—	—	—	—	—	—	—	—	—	—	—	—	—
7.44	1.2	—	—	—	—	—	—	—	3.7	—	—	—	5.2
7.94	—	—	—	—	—	—	—	—	—	—	—	—	—
8.00	—	—	—	—	—	5.9	—	—	3.5	—	—	1.4	1.0
8.65	72.4	56.6	62.6	86.4	80.2	6.6	2.8	—	17.2	—	—	—	—
8.73	—	—	—	—	—	—	—	—	—	—	—	—	—
9.00	—	—	—	—	—	—	—	—	—	—	—	—	—
9.69	—	—	—	—	—	—	—	—	—	—	—	—	—
9.85	—	—	—	—	—	—	—	1.0	—	—	100.0	—	—
10.45	—	—	—	—	—	—	—	—	—	—	—	—	—
11.14	—	—	—	—	—	—	—	—	—	—	—	—	—
11.73	—	—	—	—	—	—	—	—	—	—	—	—	—
12.14	—	—	—	—	—	—	—	—	—	—	—	—	—
13.82	—	—	—	—	—	—	—	—	—	—	—	—	—
14.04	—	—	—	—	—	—	—	—	—	—	—	—	—

13. *Stanhopea impressa* and *Stanhopea* aff. *impressa* are qualitatively and quantitatively different.

14. *Embreea* (*Stanhopea*) *rodigasiana* produces large amounts of several unique, unidentified compounds.

A list of the known visitors and pollinators of the species examined in this study is presented in Table IV. The floral fragrances of *Stanhopea* attract fragrance-col-

TABLE III (*Continued*)

	23. *S. impressa*	24. *S.* aff. *impressa*	25. *S. tigrina*	26. *S. ecornuta*	27. *S. pulla*	28. *S. pulla*	29. *S. annulata*	30. *S. annulata*	31. *S. anfracta*	32. *S. candida*	33. *Embreea rodigasiana*
4.60	—	—	—	—	1.0	—	—	—	—	—	—
4.75	3.4	—	—	—	—	—	—	1.7	1.1	—	—
4.77	—	—	—	1.5	3.2	1.0	—	—	—	—	—
4.79	—	1.6	—	—	—	—	—	—	—	—	—
4.96	—	—	—	1.1	4.2	—	—	—	—	—	—
4.99	—	—	—	—	—	—	—	—	—	—	—
5.01	—	—	—	—	—	—	—	—	—	—	—
5.51	13.6	5.7	—	20.5	—	—	3.8	23.3	37.0	—	—
5.93	1.3	40.5	2.0	1.8	41.5	4.2	—	—	—	1.1	—
5.97	—	—	—	—	—	—	3.7	—	9.3	—	—
6.04	—	—	—	—	—	—	—	—	—	—	—
6.05	—	37.1	—	66.2	18.1	11.1	3.2	1.2	1.0	1.0	4.9
6.07	38.7	—	—	—	—	—	3.0	—	—	—	—
6.25	—	—	—	1.6	—	—	—	—	—	—	—
6.27	—	—	—	—	1.0	—	—	—	—	—	—
6.68	—	—	—	—	—	—	—	—	—	1.5	—
6.70	—	—	—	—	—	—	—	—	—	—	—
6.73	—	—	—	—	5.3	—	—	—	—	—	—
6.74	—	—	—	—	—	—	—	—	—	—	—
6.75	—	—	—	—	—	—	—	—	—	—	—
6.78	—	—	—	—	—	—	—	—	—	—	—
6.79	—	—	—	—	—	—	—	—	—	—	—
6.84	—	—	—	—	—	—	—	—	—	—	—
6.89	—	—	—	—	—	—	—	—	—	—	—
6.95	7.0	—	6.5	—	—	—	9.4	2.5	—	—	—
6.97	—	—	—	—	—	—	—	—	—	—	—
7.33	—	—	—	—	3.0	8.7	2.2	—	1.8	—	—
7.40	—	—	—	—	—	—	22.7	26.6	40.3	—	—
7.44	1.4	—	1.0	—	17.6	68.4	3.2	8.1	—	—	—
7.49	—	—	—	—	—	—	—	—	—	—	6.5
7.89	—	—	—	—	—	—	—	—	—	—	—
7.91	—	—	—	—	—	1.5	—	—	—	—	—
7.94	—	—	—	—	—	—	—	—	—	88.0	—
8.00	—	1.1	—	—	—	—	—	—	—	—	—
8.65	27.7	—	69.4	—	—	—	35.0	36.7	—	—	—
8.73	—	—	—	—	—	—	—	—	—	—	—
9.00	—	9.9	3.4	—	—	—	—	—	—	6.3	—
9.69	—	—	1.0	—	—	—	—	—	—	—	—
9.85	—	—	—	1.0	—	—	—	—	—	1.0	4.9
10.45	—	—	15.8	—	—	—	—	—	—	—	—
11.14	—	—	—	—	—	—	—	—	—	—	2.1
11.73	—	1.4	—	—	—	—	—	—	—	—	—
12.14	—	—	—	—	—	—	—	—	—	—	52.7
13.82	—	—	—	—	—	2.0	—	—	—	—	—
14.04	—	—	—	—	—	—	—	—	—	—	15.8

lecting male euglossine bees to the flowers. It should be possible to explain differences in the visitors to different Stanhopeas in terms of differences in floral fragrance composition.

TABLE IV

Known visitors and pollinators of the Stanhopea *species examined in this study*

Orchid	Euglossine visitors and pollinators[a]	Citation[b]	Known chemical attractants[c] for bees
Embreea rodigasiana	unknown		
Stanhopea anfracta	unknown		
Stanhopea annulata	! *Euglossa grantii* Cheeseman	D	11
Stanhopea candida	! *Euglossa chlorosoma* Cockerell	D	1, 2, 4, 5, 7, 8, 12
Stanhopea costaricensis	p *Eufriesea rufocauda* (Kimsey)	H	1, 4
	p *Eufriesea schmidtiana* (Friese)	F	1, 5, 7, 8
	! *Eulaema luteola* Moure	E	—
	! *Eulaema meriana* (Oliver)	F	1, 2, 5, 6, 7, 8, 19
	! *Eulaema nigrita* Lepeletier	A	1, 3, 5, 8, 9, 13
	! *Eulaema seabrae* Moure	G	—
Stanhopea ecornuta	! *Eufriesea schmidtiana* (Friese)	F	1, 5, 7, 8
	! *Eulaema nigrita* Lepeletier	B	1, 3, 5, 8, 9, 13
	! *Eulaema seabrae* Moure	B	—
	n *Euglossa allosticta* Moure	F	1, 3, 5
	n *Euglossa imperialis* (Cockerell)	F	1, 2, 4, 7, 19
	n *Euglossa tridentata* Moure	F	1, 2, 3, 4, 5, 6, 7, 8, 19
Stanhopea embreei	! *Eulaema bomboides* Friese	D	5
Stanhopea gibbosa	! *Eulaema meriana* (Oliver)	B	1, 2, 5, 6, 7, 8, 19
Stanhopea impressa	! *Euglossa grantii* Cheeseman	D	11
Stanhopea oculata	! *Eufriesea caerulescens* (Lepeletier)	E	1, 4, 11
Stanhopea panamensis	p *Eufriesea ornata* (Mocsary)	H	1, 2, 4, 8, 19
	? *Eufriesea mussitans* (Fabricius)	I	8
	n *Euglossa crassipunctata* Moure	E	1, 2, 3, 4, 5, 6, 7
	n *Euglossa cyanaspis* Moure	E	1, 3, 6, 8, 19
	n *Euglossa deceptrix* Moure	E	1, 3, 5
	n *Euglossa despecta* Moure	E	1, 3, 4, 5, 6, 8, 19
	n *Euglossa hemichlora* (Cockerell)	E	2, 4, 19
	n *Euglossa tridentata* Moure	E	1, 2, 3, 4, 5, 6, 7, 8, 19
Stanhopea pulla	! *Euglossa asarophora* Moure	H	1, 4, 5, 6, 12
Stanhopea ruckeri	unknown		
Stanhopea tigrina	! *Eufriesea caerulescens* (Lepeletier)	E	1, 4, 11
	? *Euglossa viridissima* Friese	J	1, 4
Stanhopea wardii	p *Eufriesea chrysopyga* (Mocsary)	H	1, 5
	! *Eufriesea concava* (Friese)	C	1, 6, 7, 8, 11, 13, 15, 19
	p *Eufriesea rufocauda* (Kimsey)	H	1, 4
	! *Eulaema polychroma* (Friese)	B	1, 6, 8, 16, 17, 18

[a] ! = observed pollinating; p = bee captured carrying pollinaria; n = nonpollinating visitor; ? = role of visitor (pollinator or nonpollinator) uncertain.

[b] Literature citations for visitor data: A = Ackerman, in press; B = Dodson, 1965; C = Dodson, 1975a; D = Dodson, 1975b; E = Dodson, Dressler, and Williams, unpub.; F = Dressler, 1968; G = Dressler, 1979; H = Dressler, unpub.; I = Kimsey, 1982; J = Van der Pijl and Dodson, 1966.

[c] Chemical attractants for male euglossine bees (sources: Dodson, Dressler, and Williams, unpub.; Ackerman, unpub.; Kimsey, 1982.). 1—1,8 cineole; 2—methyl salicylate; 3—skatole; 4—eugenol; 5—methyl cinnamate; 6—beta-ionone; 7—benzyl acetate; 8—vanillin; 9—linalool; 10—2-phenylethyl alcohol; 11—2-phenylethyl acetate; 12—myrcene; 13—carvone; 14—menthone; 15—alpha-pinene; 16—piperonal; 17—thujone; 18—indole; 19—benzyl benzoate.

Dressler (1968) pointed out that pollination by fragrance-seeking male euglossine bees (androeuglossophily) provides a situation in which sympatric speciation might occur. An individual which produces a unique fragrance might attract a different set of euglossine pollinators, thereby resulting in ethological isolation from other individuals. Possible selfing and inbreeding could lead to stabilization of that unique fragrance. Alternatively, a morphologically uniform species might radiate into different fragrance forms in different parts of its range, perhaps in response to differences in the available euglossine faunas. Subsequent intermixing of the fragrance forms could result in the sympatry of morphologically identical but ethologically isolated sibling species. Such sibling species have been documented in *Gongora* (Dodson *et al.*, 1969; Whitten and Williams, unpubl.). Geographic variation in the fragrance of *Stanhopea tricornis* was reported by Dodson *et al.* (1969). The fragrance variations among the three samples of *Stanhopea costaricensis* presented above are indicative of different fragrance forms.

Caution should be used in drawing conclusions from the available data on fragrances and pollinators for several reasons. First, we do not have both pollination and fragrance data for individual plants; until the range of variation within species is better known, it seems unwise to link pollination data from one individual with fragrance data from another. Second, data on fragrances and pollinators of *Stanhopea* are scanty and are often based on one or a few observations per species. Finally, some of the fragrance compounds possess stereoisomers (enantiomers), but their configurations have not been determined in the floral fragrances.

Whether observed variation in fragrance composition is biologically significant can only be determined by field experiments with live plants and with various mixtures of fragrance chemicals. There is no reason to assume that all components of a fragrance are critical to the attraction of pollinators. Some compounds act as primary attractants, while others modify their attraction potential, with the resultant attraction of only a few bee species (Williams and Dodson, 1972). Other compounds might have little or no effect on the attraction of pollinators and represent biochemical noise in the system.

Several of the *Stanhopea* species examined in this study are known to produce occasional natural hybrids. *Stanhopea annulata* and *S. impressa* are both pollinated by *Euglossa grantii* and rare hybrids are found along the western slopes of Ecuador and Colombia (Dodson, pers. comm.). The main isolating mechanism between the two species appears to be mechanical. The flowers of the hybrids are morphologically altered from either parent and the insect is not able to effect pollination; therefore, no genetic material is transferred from one species to the other and the integrity of each species is maintained. A secondary isolating mechanism appears to be geographical. *Stanhopea annulata* usually occurs from 100 to 600 meters in elevation, while *S. impressa* is usually found at 700–1500 meters (Dodson, 1975b). The fragrances of both species contain large amounts of phenylethyl acetate, which is the only known attractant for *Euglossa grantii.*

Hybridization also occurs between *Stanhopea ecornuta* and *S. costaricensis* in Central America. These species share three pollinators in common (*Eulaema seabrae, El. nigrita,* and *Eufriesea schmidtiana*). Based on the available data, cineole is the only major attractant common to both Stanhopeas. Cineole attracts a majority of euglossine species, but the modifier effects of other compounds combined with cineole are poorly known. The variation in fragrances among the three samples of *S. costaricensis* is surprising, and its significance is unknown. Four species of *Eulaema* and two of *Eufriesea* are reported to visit *S. costaricensis* (Table IV); perhaps this large number of potential pollinators reflects several fragrance varieties within this species. The hybrids between *S. ecornuta* and *S. costaricensis* are morphologically altered from the parental species, and the pollinators are unable to effect pollination (Dodson, pers. comm.). More detailed study is clearly needed.

Stanhopea wardii and *S. oculata* have fragrances which are qualitatively but not quantitatively similar; both contain p-cymene, cineole, and phenylethyl acetate. *Stanhopea wardii* is dominated by phenylethyl acetate and phenylethyl alcohol, while *S. oculata* is dominated by cineole and p-cymene. P-cymene is not known to attract any euglossine bees, although it is common in orchid fragrances, and the attraction potential of cineole/p-cymene mixtures is unknown. These two species are not known to hybridize, and their fragrances are apparently dissimilar enough to attract exclusive sets of pollinators.

The fragrance of *Stanhopea panamensis* contains large amounts of benzyl benzoate, and is the only *Stanhopea* known to attract *Eufriesea ornata*. Ackerman (1983 and pers. comm.) baited for bees extensively in central Panama using benzyl benzoate, and found that, overall, benzyl benzoate is a poor attractant of 15 species of euglossines, but that it is one of the best attractants of *Eufriesea ornata*. Benzyl benzoate appears to be the primary attractant of the pollinator of *Stanhopea panamensis,* and other chemicals in its fragrance (methyl salicylate, methyl benzoate) probably reduce the number of bee species attracted, with the resultant unique set of pollinators.

Stanhopea embreei is unique in producing a fragrance composed of pure methyl cinnamate (Williams and Whitten, 1982). This compound is the only known attractant of its pollinator, *Eulaema bomboides*. This bee also pollinates *Stanhopea frymirei* Dodson, but the plant species are allopatric and hence geographically isolated. The fragrance composition of the latter species is unknown.

Stanhopea rodigasiana was recently removed from *Stanhopea* on the basis of its distinctive floral and vegetative morphology and now forms the monotypic genus *Embreea* Dodson. The fragrance of this species contains several unique unidentified compounds not known from any other orchids, thereby supporting its separation from *Stanhopea*. Its pollinator is not known.

The fragrance of *Stanhopea impressa* (#23) is dominated by phenylethyl acetate, benzyl alcohol, and myrcene, and its pollinator, *Euglossa grantii,* is occasionally attracted to pure phenylethyl acetate. *Stanhopea* aff. *impressa* (#24) produces p-cymene, cineole, indole, and myrcene. No pollinator data are available, but such a striking difference in fragrance composition suggests that it might not be pollinated by *Euglossa grantii*. This plant was collected in southern Ecuador, outside the known range of true *Stanhopea impressa,* and differs morphologically from the latter in several details of floral structure. Dodson (pers. comm.) suggests that this plant bears only superficial resemblance to *S. impressa,* and may be more closely related to other taxa.

Stanhopea tigrina exists in at least two varieties which are probably adapted to different pollinators. One form occurs in northeast Mexico and is pollinated by *Eufriesea caerulescens*. The channel formed by the tips of the column and epichile (apex of the labellum) is relatively wide, presumably to accommodate its large pollinator. The flowers are mottled with purple, and the fragrance is somewhat pungent due to the presence of indole. The second form, corresponding to *Stanhopea nigroviolacea* Morren. ex Beer., has flowers which are heavily blotched with dark purple, and the channel between the epichile and column is much narrower, perhaps to accommodate a smaller pollinator. The type specimen of *Euglossa viridissima* Friese was reportedly collected at flowers of *Stanhopea tigrina,* and this bee might be the pollinator of this southern form. *Euglossa viridissima* does not visit true *Stanhopea tigrina*. Unfortunately, neither pollination nor fragrance data are available for *S. nigroviolacea*.

The data presented above are generally consistent with the hypothesis that different species of *Stanhopea* usually produce distinct floral fragrances which results in the selective attraction of different species of euglossine pollinators. A detailed, functional understanding of the relationship is still not possible; given a particular fragrance composition, we cannot yet predict which euglossine species will be attracted. We lack adequate data on which bees are attracted to pure fragrance compounds and especially to mixtures of compounds.

Future studies of *Stanhopea* pollination should try to include both pollination observations and fragrance analysis for individual plants from known localities. Studies of intra- and interpopulational variation in fragrances are clearly needed, but are difficult due to the scarcity of plants in cultivation (and often in the field). Similarly, field studies of pollination are hampered by the rarity of flowering plants and by the short duration of the flowers. Perhaps the most profitable means of studying orchid/euglossine relationships will be to analyze fragrances of cultivated plants from known localities, and then prepare matching synthetic fragrance mixtures for use in field tests of attractiveness to male euglossine bees. This technique requires positive identification (and often chemical synthesis) of the major fragrance compounds, a goal still lacking for many of the orchid species we have sampled to date. Such synthetic fragrances are not a substitute for observation of pollination, but might prove useful in discovering the visitors of numerous orchids and other plants whose euglossine pollinators are currently unknown.

Correction and confirmation of identifications in the Catasetinae

The fragrances and pollinators of *Catasetum* (sensu lato) were surveyed by Hills *et al.* (1972). Although our collection of living plants from that study was largely dispersed in the intervening years, we have been attempting to reexamine the taxa treated in that paper, using improved fragrance analysis techniques. The following section presents corrections and additional data on fragrances of the Catasetinae.

The genus *Catasetum* consists of approximately 70 species found throughout the American tropics. With the recent segregation of *Clowesia* and *Dressleria* from *Catasetum* (Dodson, 1975c), the genus becomes much more homogeneous. One distinctive group within *Catasetum* is the *C. maculatum* complex, a group of at least nine species. Hills *et al.* (1972) stated that the fragrances of all members of the *maculatum* complex are essentially identical and are composed largely of alpha-pinene with small amounts of benzyl acetate, carvone, cineole, and other compounds. We have recently sampled a number of plants in the *C. maculatum* complex, and the results are diagrammed in Figure 2 a–j. Although some intraspecific and interspecific variation is evident, the fragrances within this sample of the complex are remarkably similar. Most of the samples are dominated by benzyl acetate, p-cymene, limonene, carvone, and an unknown compound (#16). Alpha-pinene appears to be a minor component, contrary to the earlier report. All members of the *C. maculatum* complex are pollinated primarily by *Eulaema meriana, Eulaema cingulata,* and *Eulaema polychroma.* Also included in Figure 2 are *Catasetum longifolium* and *Dalechampia spathulata. Catasetum longifolium* is not closely related to the *C. maculatum* complex, but it has a similar fragrance, attracts the same pollinators, and is reproductively isolated from sympatric species of the *C. maculatum* complex by placing the pollinarium on the underside of the bee's thorax rather than on the scutum. It is ecologically isolated by its restriction to *Mauritia* palms as hosts (Dodson, 1978). *Dalechampia spathulata* (Euphorbiaceae) is one of the few androeuglossophilous dicotyledons, and is pollinated by *Eulaema polychroma, E. cingulata,* and *E. luteola* (Armbruster and Webster, 1979). *Dalechampia*

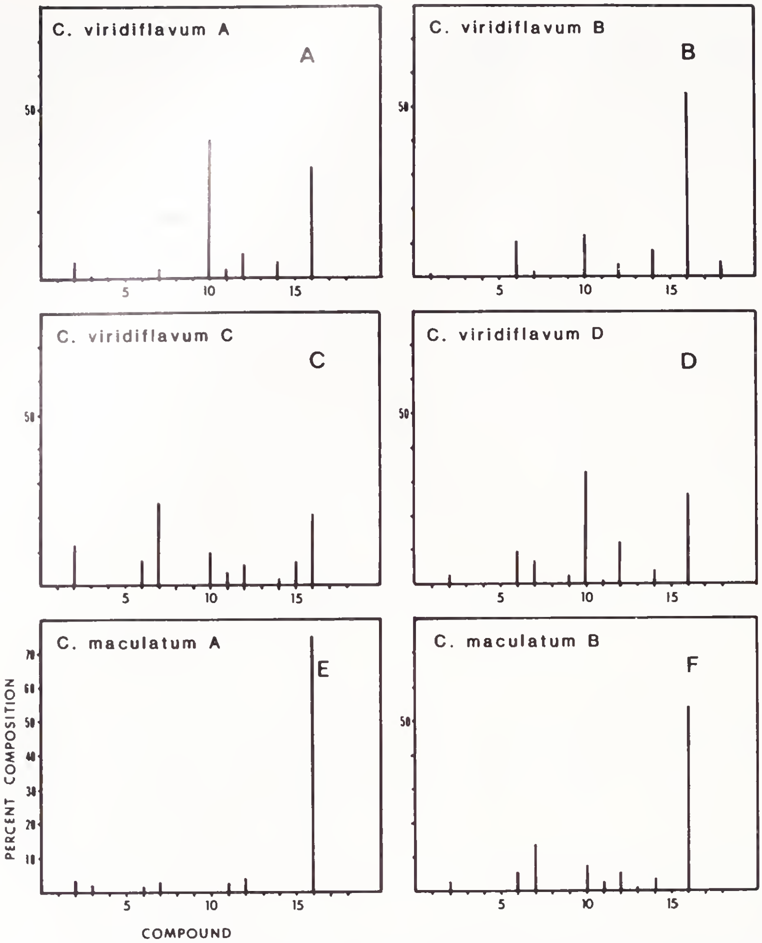

FIGURE 2, A–L. Fragrance composition of selected members of the *Catasetum maculatum* complex (A–J), *Catasetum longifolium* (K), and *Dalechampia spathulata* (L), a member of the family Euphorbiaceae. Percent composition was determined by peak areas of chromatograms. Identification of compounds: 1 = benzaldehyde; 2 = alpha-pinene; 3 = camphene; 4 = myrcene; 5 = alpha-phellandrene; 6 = p-cymene; 7 = limonene/cineole (only partly resolved); 8 = methyl benzoate; 9 = dimethyl styrene; 10 = benzyl acetate; 11 unidentified; 12 = dihydrocarvone; 13 unidentified; 14 = carvone; 15 = phenylethyl acetate; 16 unidentified epoxide MW = 166; 17 unidentified sesquiterpene; 18 = benzyl benzoate; 19 = methyl cinnamate.

spathulata and *C. longifolium* and the *C. maculatum* complex all appear to have converged upon a similar fragrance composition and hence share a common set of pollinators. Compound #16, which appears to be an epoxide related to carvone, is a major component of all the fragrances, and should prove to be a general attractant for a number of species of *Eulaema*. The other compounds present in the fragrances probably act as modifiers which restrict the numbers of *Eulaema* species attracted to the mixture. Although floral fragrances may provide characters useful in delimiting ethologically isolated sibling species of orchids, they are probably not useful in determining relationships above the species level due to the likelihood of convergence.

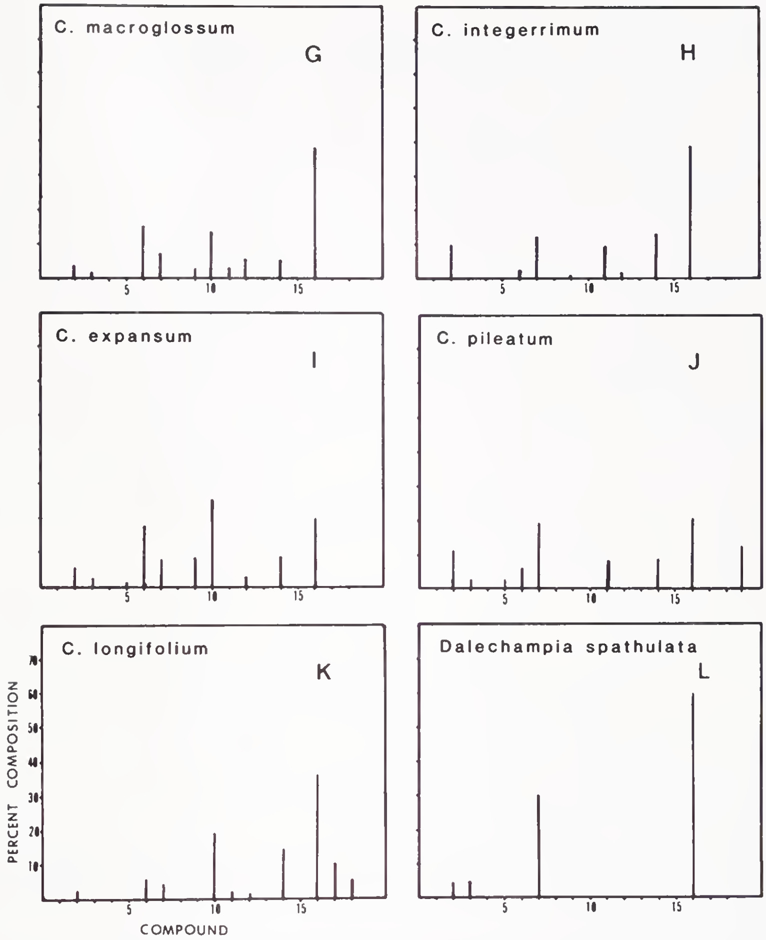

FIGURE 2 (*Continued*)

The genus *Clowesia* was recently segregated from *Catasetum* by Dodson (1975c). In 1972 we reported on the fragrances of four species that are now included in *Clowesia,* but which at that time were included in *Catasetum.* We have been able to obtain material of three of the four original species, and have data derived from gc/ms analyses of the fragrances of these three species. In *C. russelliana* we reported the presence of cineole as the major component of the fragrance. We have confirmed this with gc/ms. We have also confirmed the presence of alpha-pinene, and in addition we have found that the fragrance contains camphene, myrcene (which we thought lacking), p-cymene, phenylethyl alcohol (again, thought to be lacking), phenylethyl acetate (also not reported previously), and benzyl benzoate (also not reported). This is not surprising, since many of the late eluting compounds were very difficult to detect using the older headspace sampling technique.

In *Clowesia thylaciochila* we had previously reported that the major component was phenylethyl acetate; however, we are now quite sure that we were in error in our identification of this compound in the fragrance of this species. Instead, the compound is phenylpropyl acetate, which differs from the former compound in having an additional CH_2 in the side chain on the benzene ring. We have so far not confirmed the presence of phenylethyl alcohol in the fragrance of this species, and we have no reason at this time to believe that we will find this compound in the fragrance of *C. thylaciochila.* We have found benzyl acetate, cinnamaldehyde, indole, cinnamyl alcohol, and cinnamyl acetate in this fragrance also.

We had earlier suggested that because of the presence of phenyl ethyl acetate in the fragrance of *C. thylaciochila* that the probable pollinator might be *Eufriesea concava,* a species known to be strongly attracted to phenylethyl acetate. We are not able to make any predictions on the pollinator of this species, since we have not had the opportunity to test phenylpropyl acetate in field bioassays yet (this compound was identified as this paper was being written). The lack of detecting cinnamyl acetate is easily explained by the fact that this compound is a very late eluting peak on carbowax columns, which we had been using for the headspace analyses, and it is only with higher temperature, non-carbowax long capillary columns that we have been able to detect such compounds. Furthermore, such compounds are not so volatile as the faster eluting peaks, and headspace sampling is not the preferred method of sampling for this type of compound. We are eagerly awaiting the opportunity of testing phenylpropyl acetate and cinnamyl acetate in field bioassays in the very near future.

In *Clowesia warczewitzii* we have identified a number of previously unidentified compounds, and have one compound not previously found in orchid floral fragrances, ipsdienol. We confirm the presence of myrcene; p-cymene (previously unidentified); limonene; beta-ocimene (and an isomer of ocimene); alpha, p-dimethyl styrene; alpha-terpinene; terpinolene; nerol; and isoelemicin in the fragrance of *C. warczewitzii.* We suspect that the abundant unidentified compound we reported in 1972 is actually ipsdienol. We have not confirmed the presence of cineole in the fragrance of this species. The known pollinator of this plant is *Eulaema bombiformis,* which is attracted to cineole, but not to any of the compounds confirmed in the fragrance of this plant. We suspect that the bee will be attracted to some of the compounds in the fragrance but which we have not had the opportunity to subject to field assays.

Dressleria suavis (a segregrate from *Catasetum,* Dodson 1975c) contains methyl benzoate, methyl salicylate, phenylethyl acetate, eugenol, and benzyl benzoate in its fragrance. Unfortunately, we have not been able to obtain material of the other species of *Dressleria,* and therefore have no basis of comparison with our previously reported work.

Collection, storage, and utilization of floral fragrance compounds by male bees

Our efforts to determine the fate of the fragrance chemicals collected by male euglossine bees have centered around the hypothesis that the chemicals serve as precursors for courtship or territorial pheromones. Preliminary to testing this hypothesis, we are currently analyzing the chemicals present in the hind tibial organs and the mandibular glands of as many euglossine species as possible. Preliminary work indicated that male euglossines have large mandibular glands and associated reservoirs and produce abundant secretions, while the mandibular glands of females contain very little. It seemed reasonable to suspect the mandibular gland secretions are somehow involved in sexual behavior.

Samples are obtained by collecting male bees at various fragrance baits, removing the hind tibia and the head, and extracting the parts in separate vials of hexane.

The resulting solutions are analyzed using capillary gc/ms. Such analyses show that the mandibular glands contain a variety of compounds, usually normal alkanes, alkenes, dienes, acetates, and alcohols. The composition of the head extract is highly consistent within a species, and displays great variation between species.

The hind tibia contain two sets of compounds; one set is more or less identical to the set found in the mandibular glands, and the second set consists of various fragrance compounds (mainly mono- and sesquiterpenes and aromatics). The tibial extract is often highly fragrant, reminiscent of some perfumes. Many of the compounds that occur in orchid fragrances can be found in the hind tibia of the bees that visit the orchids. This set of fragrance compounds shows considerable variation from bee to bee (qualitative and quantitative). Presumably, the contents of the tibial organs reflect the varied sources that the bees visit to collect chemicals, and probably also varies with the age and metabolic activity of the bee. Similar extracts of the thorax and abdomen contain only trace quantities of alkanes and alkenes. Table V presents the mandibular gland compounds and their distribution in a number of *Eulaema* and *Euglossa* species.

An example of the compositions of a floral fragrance and head and tibial extracts is shown in Figure 3. The figures are total ion chromatograms of the respective samples. Figure 3A shows a fragrance sample of *Gongora quinquenervis* from El Valle de Anton, Panama. At this site *G. quinquenervis* is avidly visited and pollinated only by *Euglossa deceptrix*. The fragrance is dominated by beta-ocimene and methyl-p-methoxycinnamate. Figure 3B shows a chromatogram of the hind tibia of a specimen of *E. deceptrix*. The individual bee was collected at *G. quinquenervis* flowers. The tibial extract contains methyl-p-methoxycinnamate, benzyl benzoate, several unidentified compounds, and a large amount of eicos-10-enyl-1, 20-diacetate. The head extract shown in Figure 3C contains none of the floral fragrance compounds, but it does contain large amounts of the diacetate.

The complexity of the extracts ranges from a single compound in *Euglossa sapphirina* to nearly twenty in *Eulaema cingulata*. Some of the compounds have not been completely identified; the position of double bonds is not known for many of the unsaturated compounds. We should soon complete the chemical determinations, and hope to extend the survey to include about 50 species.

Even in this limited sample, some taxonomically interesting patterns are present at the generic level. *Eulaema* secretions are complex, with large amounts of alkanes, alkenes, and acetates. *Euglossa* secretions are usually dominated by eicosenyl-1, 20-diacetate, with one or a few other compounds present. *Eufriesea* is not included in the table, but contain alkanes, alkenes, and a distinctive set of compounds not found in the other genera. *Euglossa intersecta* is morphologically atypical for the genus and resembles *Eufriesea* in a number of characters, but its mandibular glands contain the diacetate common to most *Euglossa*.

Euglossine species differ markedly in their preferences for various fragrance chemicals. Dressler (1982) listed the attractiveness of eight chemicals to 36 species of Panamanian *Euglossa*. Some bees are not attracted to any known baits; others are attracted to only a few (*e.g. Euglossa cyanura* to p-cresol), but most are strongly attracted to two or three compounds. Cineole appears to be the best known attractant (in terms of numbers of individuals and species), with methyl salicylate, skatole, vanillin, and eugenol also ranked highly. If there is a functional relationship between the fragrances that a bee species collects and the chemicals in its mandibular glands, then one might expect the presence of a given mandibular gland compound to be correlated with a preference for a certain chemical or set of chemicals in euglossines. From the available data, it seems that the mandibular gland contents are not a good

TABLE V

Tentative identifications of compounds found in the cephalic extracts of males of various species of euglossine bees

Name (molecular weight)	#C	*El. cingulata*	*meriana*	*nigrita*	*Eg. allosticta*	*asarophora*	*cyanaspis*	*cyanura*	*cybelia*	*deceptrix*	*dissimula*	*dressleri*	*flammea*	*gorgonensis*	*hansoni*	*heterosticta*	*imperialis*	*intersecta*	*maculilabris*	*mixta*	*sapphirina*	*tridentata*	*villosiventris*
alkanes																							
n-heneicosane (296)	C-21	+	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-tricosane (324)	C-23	+	+	+	—	*	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-tetracosane (338)	C-24	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-pentacosane (352)	C-25	+	*	+	—	*	*	*	*	*	—	*	—	—	—	—	—	—	—	—	—	*	—
n-heptacosane (380)	C-27	*	*	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-nonacosane (408)	C-29	—	—	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
alkenes																							
n-tetradecene (196)	C-14	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-heneicosene (294)	C-21	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-tricosene (322)	C-23	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-pentacosene (350)	C-25	+	*	*	—	+	—	*	—	—	—	*	—	—	—	—	—	*	*	—	—	—	—
n-heptacosene (378)	C-27	+	*	—	—	+	—	*	+	—	—	+	*	*	—	—	*	—	—	—	—	—	—
n-octacosene (392)	C-28	*	—	—	—	—	—	—	—	—	—	—	*	—	—	—	—	—	—	—	—	—	—
n-nonacosene (406)	C-29	+	*	—	*	—	+	—	—	*	—	—	—	—	—	—	+	—	—	—	—	*	*
n-hentriacontene (434)	C-31	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
dienes																							
n-docosadiene (306)	C-22	—	—	—	—	—	—	—	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-heptacosadiene (376)	C-27	—	—	—	—	—	—	+	—	—	—	—	—	—	*	—	—	—	—	—	—	—	—
n-nonacosadiene (404)	C-29	+	—	—	—	—	—	*	—	—	—	+	—	—	*	—	—	—	—	—	—	—	—
n-hentriacontadiene (432)	C-31	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-tritriacontadiene (460)	C-33	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

acetates																							
dodecyl-1-acetate (228)	C-14	+	+	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
tetradecyl-1-acetate (256)	C-16	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
hexadecyl-1-acetate (284)	C-18	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
octadecyl-1-acetate (312)	C-20	+	+	+	—	*	+	—	—	—	—	—	—	—	—	—	—	—	—	*	—	*	—
hexadecenyl-1-acetate (282)	C-18	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
octadecenyl-1-acetate (310)	C-20	—	—	—	—	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
eicosenyl-1-acetate (338)	C-22	—	—	—	+	+	—	—	—	—	*	—	—	—	—	*	—	—	—	+	—	+	—
docosatrienyl-1-acetate (394)	C-24	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—
diacetates																							
eicos-10-enyl-1,20 diacetate (396)	C-24	—	—	—	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	*	—	+	—
tetracosanyl-1,24-diacetate (454)	C-28	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
alcohols																							
octadecen-1-ol (268)	C-18	—	—	—	+	—	—	—	*	—	—	—	—	—	*	—	—	*	—	—	—	—	—
eicosadien-1-ol (294)	C-20	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
other compounds (unidentified)																							
mw = 240 (#1)		—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
mw = 240 (#2)		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*
mw = 276		—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—
mw = 290		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
mw = 312 (#1)		—	—	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
mw = 312 (#2)		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*	—	—	—	—	—
mw = 318		—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+
mw = 327 (±)		—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
mw = 340		—	—	—	—	—	—	—	—	—	—	—	*	—	—	—	—	—	—	—	—	—	—
mw = 478		—	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

+ = a major compound.
* = a minor compound, — = not present.

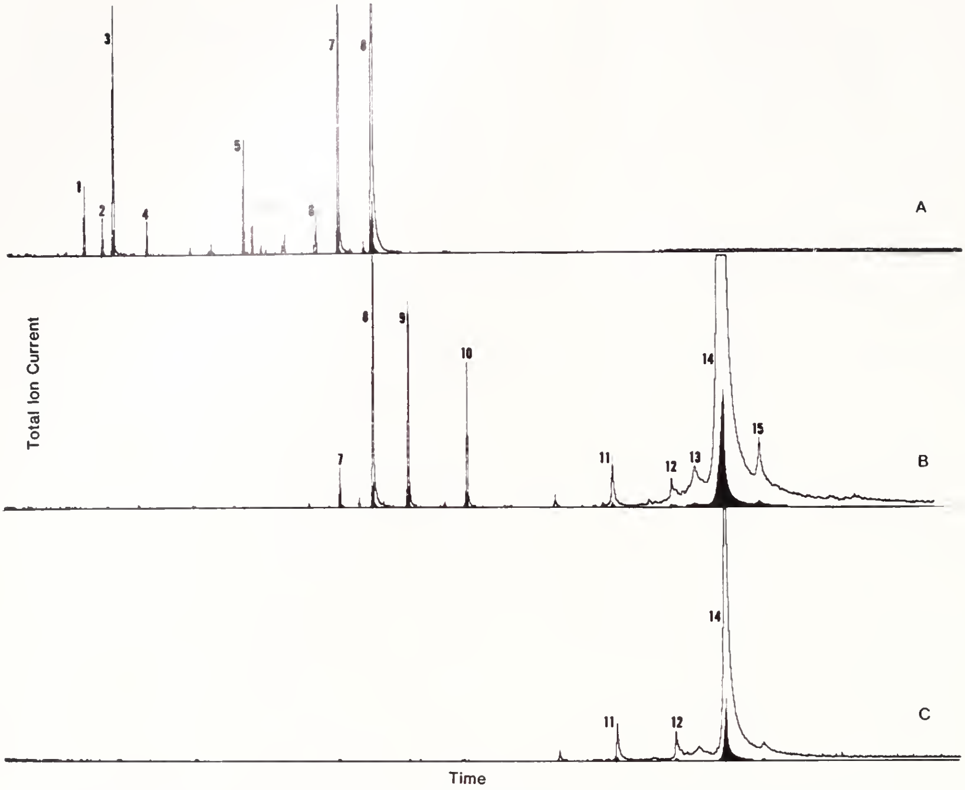

FIGURE 3, A–C. Total ion chromatograms of the floral fragrance of an orchid and extracts of the bee that pollinates it. A. Total ion chromatogram of the fragrance of *Gongora quinquenervis* from El Valle de Anton, Panama. B. Total ion chromatogram of hind tibial extract of *Euglossa deceptrix*, the pollinator of *G. quinquenervis* at El Valle. C. Total ion chromatogram of the cephalic extract of the same individual bee. Note the sets of compounds shared between A and B and between B and C. See text for details. Identification of peaks: 1 = beta-ocimene; 2 = terpinolene; 3 unidentified; 4 = methylphenylacetate; 5 = eugenol; 6 = methyl cinnamate; 7 = cis-methyl-p-methoxycinnamate; 8 = trans-methyl-p-methoxycinnamate; 9 = benzyl benzoate; 10 unidentified; 11 unidentified acetate; 12 unidentified; 13 unidentified; 14 = eicos-10-enyl-1,20-diacetate; 15 = n-nonacosene.

predictor of a species' fragrance preference. One possible exception is that the few *Euglossa* species which lack the eicosenyl diacetate are not attracted to cineole.

Figure 4 summarizes the distribution of these compounds within the male bee and diagrams our hypothesis of the fate of the floral fragrance compounds. We suspect that the fragrance compounds are absorbed into the tibial organ and are metabolized there to form the long-chain alkanes, alkenes, acetates, etc. These compounds would be transported via the hemolymph (possibly via sequestration) to the mandibular glands and stored in the reservoir. Obviously, experiments using radioactively-labeled fragrance compounds will be needed to test these hypotheses. The current data can only demonstrate that the mandibular glands and the tibial organs share a common set of compounds which are often species-specific, and it still seems reasonable to suspect that the collected fragrances serve as precursors for these large compounds.

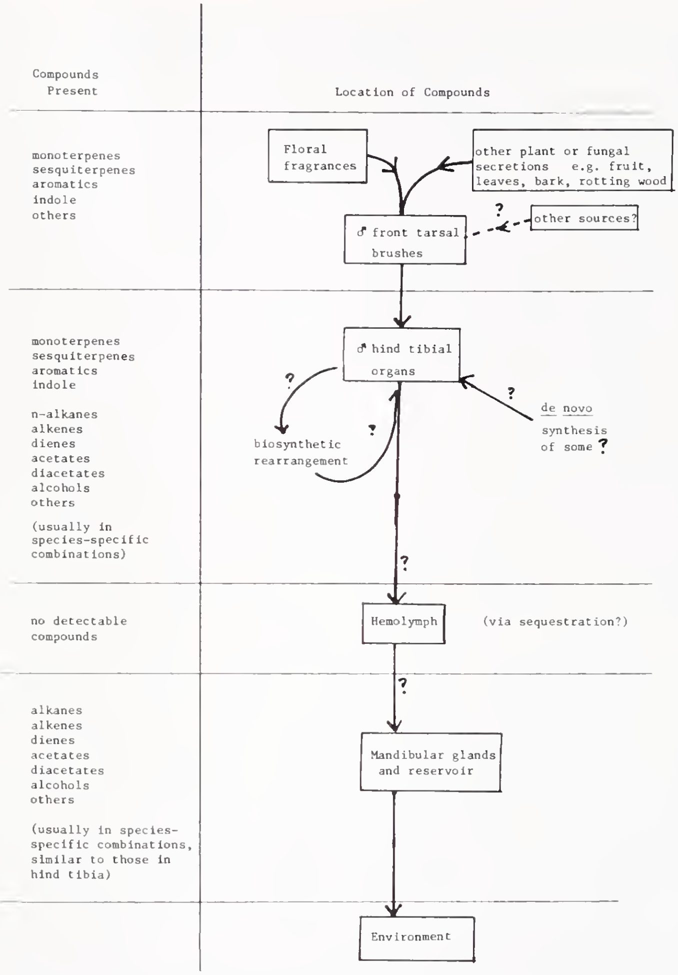
Compounds Present
Location of Compounds
monoterpenes
sesquiterpenes
aromatics
indole
others
Floral fragrances
other plant or fungal secretions e.g. fruit, leaves, bark, rotting wood
♂ front tarsal brushes
?
other sources?
monoterpenes
sesquiterpenes
aromatics
indole
n-alkanes
alkenes
dienes
acetates
diacetates
alcohols
others
(usually in species-specific combinations)
♂ hind tibial organs
?
biosynthetic rearrangement
?
?
de novo synthesis of some ?
?
no detectable compounds
Hemolymph
(via sequestration?)
?
alkanes
alkenes
dienes
acetates
diacetates
alcohols
others
(usually in species-specific combinations, similar to those in hind tibia)
Mandibular glands and reservoir
Environment

FIGURE 4. Hypothesized relationships between orchid floral fragrances and compounds occurring in hind tibial organs and mandibular glands of male euglossine bees. See text for discussion.

There have been a few reports of male *Eufriesea purpurata* brushing on surfaces treated with insecticidal sprays (van der Pijl and Dodson, 1966; Dressler, 1967; Roberts *et al.,* 1982). Roberts *et al.* reported that technical grade DDT, used for malaria control, is an excellent attractant of male *Eufriesea purpurata* in Amazonas, Brazil. Analysis of the body parts of the bees revealed astonishingly high concentrations of DDT, especially in the hind legs. It is not clear that DDT is the actual attractant of *Ef. purpurata* since pure DDT was not tested, but the results confirm that the bees can tolerate doses of DDT tens or hundreds of times greater than most insects. The report by Roberts *et al.* raises more questions than it answers, but it does suggest that the bees might be able to sequester or metabolize otherwise toxic doses of novel chemicals.

Since we still do not understand the role of the mandibular gland secretions in the euglossine life cycle, it is instructive to compare the available data on euglossines with other related bees. Male bumblebees (*Bombus*) are known to mark territorial sites with the contents of their mandibular or labial glands. The secretions attract both males and females of the same species and are thought to play some role in their mating behavior (Kullenberg *et al.*, 1970). Interestingly, a number of compounds in *Bombus* and *Psithyrus* secretions are also found in male euglossines. These include primary alkanes, alkenes, alcohols, and acetates (Bergstrom *et al.*, 1968; Kullenberg *et al.*, 1970). Some *Bombus* also produce minor amounts of geraniol, citronellol, geranyl acetate, farnesol, geranylgeraniol, and geranylcitronellol. Such compounds have not been detected in euglossine mandibular glands, but they would not be out of place in the tibial organs. Observations of male euglossines performing territorial displays are not common, and sightings of courtship and mating are rare. Males of several species are known to establish territories centered around a tree trunk (often in a tree fall clearing), to patrol and defend the area against other conspecific males, and to display on perches and mate with females that enter the territory (Kimsey, 1980). A number of male territories may be aggregated in a favorable site, such as a large tree fall, but there is no evidence to suggest that males actively form leks. Kimsey also states that the males do not open their mandibles while perching and displaying, and sees no evidence that the males mark their territory with mandibular gland secretions. She also suggests that pheromones might be used only for short-range communication and mating behavior, not for a long-range attraction of females. The high molecular weight of many of the mandibular gland compounds would support this suggestion.

It is possible that the mandibular gland compounds are used for purposes other than territoriality or mating. Anyone who has collected and handled live male euglossines, especially the larger *Eulaema* and *Eufriesea,* often notices an odd, slightly rancid odor released from the bees as they are handled. The odor is similar to that of the mandibular gland contents, and it seems likely that the bees release mandibular gland secretions when disturbed. This suggests that the odor may represent a defensive secretion or alarm pheromone. When captured, the bees attempt to bite repeatedly, opening and closing the mandibles. Since the mandibular gland duct is thought to open and close with the movement of the mandibles, this may represent nothing more than an inadvertent release of the reservoir contents. After collecting a large number of male bees, the insect net sometimes becomes tainted with the odor, yet the net seems to have little effect on the wariness of other fragrance-collecting bees. It seems improbable that an alarm pheromone would consist of a complex, species-specific mixture of large molecules of low volatility, but the secretions may serve some role in defense against predators.

Directions for future research

It is clear that many of the facets of the orchid/euglossine interaction are not well understood, and there are numerous profitable areas for further research. Even the alpha taxonomy of the two groups is incomplete. Some suggestions for future research are listed below.

1. Perhaps the most critical need is to perform tracer experiments with ^{14}C-labeled fragrance compounds. It is possible to maintain at least some species of euglossines in large flight cages for weeks or months (Ackerman, Kimsey; pers. comm.). Captive bees could be permitted to collect labeled fragrances and later sacrificed and examined for the presence and composition of labeled compounds in various body parts. We hope to attempt this in the near future with several of

the more common Panamanian *Eulaema* and *Euglossa*. The results of such experiments should help to direct subsequent studies of euglossine biology.

2. Assuming the mandibular gland compounds are functionally related to the fragrances, the chemical survey of head and tibial extracts should be extended to as many taxa as possible. Some euglossine species show interesting geographic variation in morphology and coloration; we do not know whether fragrance preferences and/or mandibular gland contents also vary geographically.

The complete characterization of mandibular gland contents should allow synthesis of individual components and allow us to field-test them singly and in combinations. This approach has been useful in studying similar problems in *Bombus* and other bees, and in the *Ophrys* pseudocopulation system.

3. We need to analyze the fragrances of as many androeuglossophilous orchids, aroids, and other plants as possible. Some of the compounds appear to be novel, and many others present a great challenge to identify with samples of a milligram or less. We expect the analyses of fragrances and hind tibia to yield a number of new attractants for male euglossines. This should accelerate the collection of new or poorly known bee species. It would also be interesting to compare fragrances of euglossine-pollinated orchids with those pollinated by insects other than euglossines.

4. Much work remains in testing various fragrance compounds as attractants. Especially needed are experiments testing the attractiveness of various isomers (of known purity) of a given compound. There are little data on geographical and seasonal variation in the bees' response to baits, and on the attractiveness of pure compounds vs. mixtures.

5. Dressler (1976, 1982) discussed the utility of fragrance baits as a tool for studying orchid pollination. A small fraction (usually 5% or less) of bees caught at baits carry the pollinaria of various orchids. Many of the pollinaria can be identified to genus and sometimes species. Since a bee carrying a pollinarium is usually a legitimate pollinator of the orchid, a great deal of information can be obtained by baiting for bees and examining the pollinaria they carry. Occasionally, a bee may carry a pollinarium that cannot be associated with any known orchids and will spur a search for an undescribed orchid species (*Sievekingia;* Ackerman, pers. comm.).

6. The inter- and intrapopulational variation in fragrance composition should be examined for a variety of orchid species. Some genera may contain numerous, poorly differentiated fragrance forms, while others may possess consistent, species-specific fragrances. We are currently studying variation in fragrances and pollinators in the *Gongora* species of central Panama, and geographic variation appears to be great.

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THE EFFECT OF FAST, AND REGENERATION IN LIGHT VS DARK, ON REGULATION IN THE HYDRA-ALGAL SYMBIOSIS[1]

PATRICIA BOSSERT AND L. B. SLOBODKIN

Department of Ecology and Evolution, S.U.N.Y., Stony Brook, L.I., New York 11794

ABSTRACT

Green hydra are able to regenerate tentacles after fast durations which cause brown, *i.e.*, asymbiotic, hydra to fail completely, but the presence of endosymbiotic algae does not always enhance regeneration in fasted hydra.

Green hydra whose nutritional state falls below some threshold, exhibit a "light induced" inhibition of regeneration. That is, hydra, fasted in the light, then randomly assigned to light or dark after decapitation, regenerate better in the dark. This effect of light does not appear to be present either in brown hydra or in normally green hydra from which the algae have been removed.

In a large strain of *Chlorohydra viridissima*, after fasts of intermediate duration (10 and 15 days), this light induced inhibition of regeneration is associated with an increase in the number of algae per gastric cell in regenerating hydra relative to non-regenerating controls.

We have not been able to associate this algal increment with an increase in hydra gastric cell mitosis for animals fasted 9 days prior to decapitation and allowed to regenerate 48 hours in the light.

INTRODUCTION

The maintenance of a symbiotic association characterized by long term stability obviously requires regulation of the numbers of one symbiont relative to the other. The mechanism for maintaining such stability will vary with the intimacy of the association, the nature of the exchange or other interactions between the symbionts, and the inherent reproductive capacities of each organism.

In a multicellular organism, when the cell itself becomes the habitat of a symbiont, one may ask: To what extent has the intracellular symbiont become an organelle of the host cell (Margulis, 1970)? This is partially reducible to another question; that is, to what degree is regulation imposed upon the symbiont by the host cell and to what degree does it emanate from other cells (*i.e.*, from the organism as a whole)?

Green hydra, whose gastrodermal cells contain Chlorella-like algae, are well suited for studying this question. With a given feeding frequency and light regime, the numbers of algae present are characteristic of both body region (Pardy, 1974) and strain (McAuley, 1979). The reproductive rate of the algae increases about 20 fold when cultured free of the host cell environment (Jolley and Smith, 1978). Under normal conditions the algae are neither digested nor expelled (Muscatine and Poole, 1979).

Regulation at the organismal level was suggested by Pardy and Heacox (1976). Removal of the hypostome and tentacles increased the number of algae in peduncle

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cells. Grafting a hypostome onto the peduncle prevented this algal increase. They suggested that a factor emanating from the hypostome directly affected algal reproduction within the host hydra cell.

Recently, McAuley (1981) repeated the experiment of Pardy and Heacox (1976). He measured changes in algae per cell in regenerating heads (tentacles, hypostome, and a collar of tissue proximal to the hypostome) and peduncles. He found an increase in the number of algae per cell in both tissues suggesting that presence or absence of a hypostome is not the critical factor affecting changes in the number of algae per hydra cell.

Further, he found a higher mitotic index in regenerating peduncle tissue than in non-regenerating controls, 48 hours after cutting. He suggested that the algae increase in number relative to host cells because they complete the mitotic cycle faster than host cells, and that the stimulus for increased mitosis in both host and algal cell is purely a local, cellular phenomenon.

Here we report the preliminary studies which caused us to become interested in the mechanism(s) by which green hydra regulate their endosymbiotic algae.

MATERIALS AND METHODS

Hydra species and maintenance of stock

Hydra used in these studies have been cultured in our laboratory for several years in a controlled temperature chamber at 20°C and in constant light according to the methods of Loomis and Lenhoff (1956). We designate all our green hydra, *Chlorohydra viridissima* (viridis), giving clones of distinct origin a strain name. The larger strain was purchased from Carolina Biological Supply Company (Carolina strain) and the smaller was isolated from the Nissequogue River near Stony Brook, New York (Nissequogue strain). The brown species used were *Hydra americana*, *Hydra oligactis*, and *Hydra cauliculata*. Aposymbiotic clones were prepared from green hydra using Pardy's method (1976).

Since experiments took place over a 5 year period, variation in the frequency with which stocks were fed occurred. However, all animals in a given experiment came from stocks with the same recent feeding history as indicated: Experiment 1 and 2—Stock animals fed 1–3 times per week; Experiment 3 and 4—Stock animals fed Monday through Friday; Experiment 5—Stock animals fed Monday, Wednesday, and Friday.

Hydra stocks are fed, *ad lib* to repletion, with 24–36 hour *Artemia salina* nauplii.

Maintenance and selection of experimental animals

The criteria for selection of animals from various stocks was as follows: Experiment 1—Animals with one small bud on which no tentacles were present; Experiment 2—The largest, non-budding individuals; Experiment 3 and 4—The largest, non-budding individuals that had actually ingested food during the last feeding prior to the onset of fast; Experiment 5—Same as experiment 2.

In all experiments animals were removed from the parent culture, placed in bowls of the same size, and maintained in the same chamber as the parent stock. In the first experiment, three nutritional levels were established as follows: Level 1—Fasted for 5 days; Level 2—Fasted for 5 days, fed 5 hours prior to decapitation; Level 3—Fed daily for 5 days. In the remaining experiments, a fasting experimental stock was established and animals were removed from it periodically for treatment.

Preparation of slides for algal counts

Hydra tissue was macerated in a deep well spot dish or directly on an uncoated glass slide in a drop of macerating fluid (David, 1973). All counts were performed on tissue comprising approximately the upper half of decapitated hydra (*i.e.*, gastric and budding regions) or the middle third of non-regenerating hydra (*i.e.*, gastric and budding regions). In those decapitated hydra where regeneration preceded maceration, newly regenerated tentacles were removed as completely as possible before the assay region was macerated.

Preparation of slides for mitotic assay

Dried gastric tissue macerates were stained for approximately 10 minutes with DAPI (4′6-diamidine-2-phenylindole di-hydrochloride) in 'M' solution (Loomis and Lenhoff, 1956) at a concentration of 1 mg/ml and examined using a Leitz epiflorescent microscope immediately after staining.

Conditions of regeneration

Regeneration occurred in the same chamber as the parent stock (constant light, 20°C). For dark regeneration, dishes containing experimental animals were placed under a cardboard box. Animals regenerating in the dark were exposed to light for a brief period daily while tentacles were counted and measured.

RESULTS

Effect of fast on degree of regeneration in brown vs green hydra: Experiment 1, Table I

Groups of 10 hydra were fed with different frequencies. Animals were decapitated and the number of animals on which tentacles regenerated after 24 and 48 hours in the light was recorded. When fasted for five days green hydra (Nissequogue) regenerated following decapitation but brown hydra (*H. oligactis, H. americana, H. cauliculata*) did not.

Effect of fast on degree of regeneration in light vs dark: Experiment 2, Tables II and III

Groups of 10 hydra were removed from stock and either decapitated immediately or fasted for 2 days and then decapitated. Five animals of each group were placed in the dark, five remained in the light and both were allowed to regenerate for 96 hours. Animals that disintegrated before 96 hours were discarded from the experiment. Animals that remained intact, but failed to regenerate by 96 hours were scored as zero.

Fed green hydra (Nissequogue) and 2 day fasted green hydra (Carolina) regenerate more tentacles in the dark than in the light. Brown hydra (*H. americana*) regenerate equally well in light and dark (Table II.)

In the last two trials of this experiment, in addition to recording the number of tentacles regenerated, the length:width ratio was also determined for each tentacle in an extended condition. For brown hydra, the length:width ratio of tentacles is identical in light and dark after 96 hours of regeneration. For green hydra, tentacles are longer in dark than in light for fed animals (Nissequogue) and for 2 day fasted animals (Carolina) (Table III).

TABLE I

Effect of fast on regeneration in brown vs green hydra

Feeding level	Hours of regeneration	*Hydra oligactis*	*Hydra americana*	*Hydra cauliculata*	Nissequogue strain
1	24	0	0	0	60
	48	0	0	0	100
2	24	0	0	10	70
	48	0	0	20	100
3	24	60	10	50	90
	48	80	20	60	90

Percent regeneration determined as the number of hydra on which tentacles were visible ÷ 10 (the total number of hydra in each group).

Changes in the number of algae per gastric cell and degree of regeneration in light vs dark as a function of fast: Experiment 3, Tables IV, V and VI

At intervals of 2, 10, 15, and 20 days, 6 hydra were removed from each fasting stock of Nissequogue and Carolina strains and decapitated. Three animals were placed in dark, three in light and all allowed to regenerate until differences between them appeared. The degree of regeneration was determined for each animal in each treatment. Gastric regions were pooled and macerated as described in Methods. Controls consisted of 3 intact animals fasted for the same number of days and placed

TABLE II

Effect of fast on the number of tentacles regenerated in light vs dark

Strain	Length of fast (days)	Light	Dark	Significance
Nissequogue	0	4.8 ± 1.4 (25)	5.6 ± 0.8 (24)	$P > .001$
	2	3.4 ± 2.8 (11)	4.3 ± 2.0 (15)	ns
Carolina	0	4.7 ± 1.8 (25)	4.3 ± 2.3 (24)	ns
	2	4.9 ± 0.6 (15)	5.9 ± 1.1 (15)	$P > .01$
H. americana	0	4.3 ± 0.9 (19)	3.2 ± 2.3 (20)	ns
	2	3.2 ± 2.1 (10)	2.6 ± 2.4 (10)	ns

Tentacles were counted on groups of five hydra after 4 days of regeneration. Replication of the experiment was as follows: FED—Nissequogue—5; Carolina—5; *H. americana*—4. FASTED—Nissequogue—3; Carolina—3; *H. americana*—2. The number of animals examined is in parentheses. The mean is expressed ± s.d. The significance of the difference between the means was determined by the Wilcoxon two sample test.

TABLE III

Length:width ratio of tentacles regenerated in light vs dark

Strain	Length of fast (days)	Light	Dark	Significance
Nissequogue	0	1.6 ± 0.7 (10)	5.3 ± 0.7 (10)	$P > .001$
	2	2.8 ± 2.4 (8)	2.8 ± 2.5 (10)	ns
Carolina	0	3.2 ± 2.0 (10)	4.7 ± 2.1 (10)	ns
	2	3.5 ± 2.5 (10)	8.1 ± 1.7 (10)	$P > .01$
H. americana	0	1.5 ± 0.7 (10)	0.9 ± 0.9 (10)	ns
	2	1.6 ± 1.0 (10)	1.0 ± 0.8 (10)	ns

Tentacles were measured in an extended condition on groups of 5 hydra after 4 days of regeneration. The mean is expressed ± s.d. The number of hydra examined is in parentheses. The significance of the difference between the means was determined by the Wilcoxon two sample test.

in either light or dark for the same time as the regenerating animals. Gastric regions from these animals were excised, pooled and macerated as above.

Degree of regeneration in light vs dark

For Carolina fasted 10, 15, and 20 days the degree of regeneration was greater in the dark. That is, Carolina regenerates marginally more ($P = .1$) and longer ($.05 > P > .025$) tentacles in dark than in light after fasts of 10, 15, and 20 days. For the smaller Nissequogue strain, those animals surviving similar conditions appeared to regenerate equally well in light and dark (Table IV).

Number of algae per gastric cell in animals regenerating in light vs dark

For Nissequogue, the only significant increase in the number of algae per gastric cell, is in animals regenerating in the light after 15 days of fast prior to decapitation

TABLE IV

Number, and length:width ratio of tentacles regenerated in light vs dark in fasted green hydra

		Light	Dark	Significance
Nissequogue	Tent/Polyp	3.1 ± 2.3	2.3 ± 2.4	ns
	L:W	2.1 ± 3.2	0.86 ± 0.90	ns
Carolina	Tent/Polyp	3.0 ± 1.7	4.2 ± 1.1	$P = .1$
	L:W	1.1 ± 0.63	3.1 ± 1.7	$.05 > P > .025$

The results of fast durations 10, 15, and 20 days are pooled. Thus, for Nissequogue in light n = 10; dark, n = 7 and for Carolina in light n = 10; dark, n = 11. The means are expressed ± s.d. Significance of the difference between means was determined by the Wilcoxon two sample test.

(Table V). This algal increase is not associated with a diminished capacity to regenerate in the light, however (Table IV). For Carolina, there is a significant increase in the number of algae per cell after both 10 and 15 days of fast for animals regenerating in the light (Table V). These same animals show a significantly lower degree of regeneration in the light (Table IV).

Degree of regeneration per polyp and number of algae per cell is shown in Table VI for Carolina. When fast durations of 10, 15, and 20 days precede decapitation, Carolina regenerates more and longer tentacles in the dark than in the light. For 10 and 15 days of fast, this light induced inhibition of regeneration is associated with a significant increase in the number of algae per gastric cell in decapitated animals relative to non-decapitated controls. For 20 days of fast, a light induced inhibition of regeneration is clear, but it is not associated with an increase in the number of algae per cell in the regenerating animals.

Effect of fast on degree of regeneration in light vs dark in aposymbiotic hydra: Experiment 4, Table VII

At intervals of 2, 10, 15, and 20 days, 6 hydra were removed from fasted aposymbiotic stocks of Carolina and Nissequogue strains and decapitated. Three animals were placed in dark, three in light, and all allowed to regenerate for 72 hours. (After 15 days of fast, Nissequogue animals appeared about to disintegrate. The degree of

TABLE V

Number of algae per gastric cell in hydra regenerating in light vs dark

Day of fast	Hours of regeneration	Strain	Light		Dark	
			Decapitated	Control	Decapitated	Control
2	72	C	15.1 ± 9.6 (3)	14.4 ± 5.8 (3)	16.6 ± 6.8 (2)	17.5 ± 7.6 (3)
		N	16.2 ± 6.8 (3)	16.0 ± 8.0 (3)	15.4 ± 9.6 (3)	16.0 ± 7.4 (3)
10	48	C	17.7 ± 8.0** (1)	14.6 ± 10.0** (3)	20.7 ± 9.3 (3)	21.2 ± 10.9 (3)
		N	23.9 ± 9.1 (3)	25.5 ± 11.4 (3)	20.7 ± 9.6 (3)	25.0 ± 11.1 (3)
15	96	C	30.2 ± 11.7*** (3)	21.1 ± 9.3*** (3)	21.0 ± 9.5 (3)	— (0)
		N	26.9 ± 13.1** (3)	21.2 ± 10.8** (1)	22.0 ± 9.0 (1)	22.8 ± 8.0 (1)
20	72	C	20.6 ± 9.0 (3)	21.7 ± 10.1 (3)	16.2 ± 9.5 (3)	22.4 ± 11.3 (3)
		N	—	—	—	—

* C = Carolina; N = Nissequogue.

The mean is expressed ± s.d. The number of hydra from which cell samples were pooled is in parentheses. For each sample n = 100 cells. Since the distribution of algal count data is not normally distributed, a *t* test was performed on log transformed data (Sokal and Rohlf, 1969).

TABLE VI

Regeneration and number of algae per gastric cell in hydra regenerating in light vs dark after a fast

	Regeneration				Number of algae per cell			
	Tent./Polyp		Length:width		Light		Dark	
Fast (days)	L	D	L	D	Dec.	Control	Dec.	Control
	—	3	—	1				
10	—	3	—	1	17.7 ± 8.0	14.6 ± 10.0	20.7 ± 9.3	21.2 ± 10.4
	0	3	0	1				
15	4	5	1	5				
	4	5	2	3	30.2 ± 11.7	21.1 ± 9.3	21.0 ± 9.5	—
	5	5	1	3				
20	2	6	1	5				
	4	4	2	4	20.6 ± 9.0	21.7 ± 0.1	16.2 ± 9.5	22.4 ± 11.3
	2	4	2	5				

The number of tentacles regenerated and the length:width ratio is presented for each of three experimental animals. A (—) indicates that an animal died before tentacles regenerated. Algal counts were performed on 100 gastric cells pooled from animals surviving the fast. A (—) indicates algal count data was lost.

regeneration in both strains was therefore recorded at 48 hours for these 15 day fasted animals.)

Data in Table VII shows that fasted, aposymbiotic hydra of either strain do not regenerate better in the dark. This is in contrast to what we found for fasted green hydra for which light is sometimes detrimental to regeneration.

Mitotic index of gastric cells in fasted hydra regenerating in light: Experiment 5, Table VIII

After 9 days, 28 animals of fasted Carolina stock were decapitated, and allowed to regenerate for 48 hours in light. The gastric regions were excised, pooled 7 to a slide, and macerated as described in Methods. Controls were gastric regions from intact hydra fasted for 11 days.

Before determining the mitotix index in fasted, regenerating hydra, we tested the DAPI staining technique developed by Muscatine and Neckelmann (1981).

TABLE VII

Regeneration in light vs dark in fasted aposymbiotic hydra

		Light	Dark
Nissequogue	Tentacle/Polyp	2.9 ± 2.4	3.3 ± 2.2
	Length:Width	3.9 ± 6.9	3.5 ± 3.9
Carolina	Tentacle/Polyp	3.6 ± 1.7	2.7 ± 1.5
	Length:Width	4.3 ± 3.4	1.0 ± 0.77

The results are pooled for 2, 10, 15, and 20 days of fast. For Nissequogue regenerating in the light n = 10; in the dark, n = 11. For Carolina regenerating in the light, n = 12; in the dark, n = 11. The means are expressed ± s.d.

TABLE VIII

Mitotic index and number of algae per gastric cell in hydra regenerating after fast

	Control		Regenerating	
Strain	Mitotic index	Algae/cell	Mitotic index	Algae/cell
Carolina	0.4	24.2 ± 9.9	0.4	25.4 ± 8.1
	0.4	23.0 ± 8.2	0.2	25.0 ± 9.8
	0	22.6 ± 11.1	0	27.6 ± 11.2
	0.4	23.6 ± 10.6	0	25.6 ± 11.7
Mean (n = 4)	0.3 ± 0.2	23.4 ± 0.7*	0.15 ± 0.7	25.9 ± 1.2*

Each of the replicates represents a slide on which the gastric regions from 7 hydra were pooled and macerated. The number of algae per cell was determined by counting algae present in 60 gastric cells. The mitotic index was determined by counting the number of mitotic figures in 500 gastric cells. The significance of the difference between the number of algae per cell in regenerating hydra vs controls was determined by t test on untransformed data, df = 3 (Sokal & Rohlf, 1969).

Under conditions designed to give maximum mitosis (10 hours after feeding) they report a mitotic index of 1.22 ± 0.16 s.e. for fed hydra and 0.54 ± 0.13 s.e. for unfed controls in the Florida strain. We repeated their procedures, except that animals were assayed 12 hours after feeding and we used Nissequogue strain. Finally, mitotic figures were identified and counted on slides without the observer knowing if she was examining fed hydra or controls. We found a mitotic index of 0.9 ± .1 s.e. in fed hydra compared to 0.4 ± .1 s.e. for controls.

Satisfied with our use of the technique, we proceeded to apply it to determine changes in the mitotic index and number of algae per cell in fasted, regenerating hydra. In Carolina strain fasted for 9 days and regenerating 48 hours in the light, the number of algae per gastric cell is significantly higher than in controls ($P = .05$). We found no increase in the mitotic index of gastric cells in these same animals.

DISCUSSION

The light-induced inhibition of regeneration is not associated with any absolute length of fast. Rather, it appears to occur under conditions where the nutritional state of the green hydra falls below some critical level *i.e.*, in 0 and 2 day fasted animals removed from stocks fed erratically (1–3 times/week) and after 10–20 days of fast in animals removed from heavily fed stocks. It is not clear if there is a strain specificity associated with this response. Under those conditions where Carolina clearly regenerates better in the dark (after fasts of 15 and 20 days: Table VI), individual Nissequogue polyps frequently failed to regenerate.

When algal endosymbionts are removed from hydra, the detrimental effect of light on regeneration is also removed (Table VII). There is some indication that light-induced inhibition of regeneration is associated with an increase in the number of algae per cell in the regenerating hydra, at least for the larger strain, Carolina (Table VI). For the smaller, Nissequogue strain, an algal increase in regenerating hydra is found only after 15 days of fast and no light induced inhibition of regeneration is associated with it (Table V).

Fasting green hydra show a net accumulation of glycogen and a gradual catabolism of protein (Cook and Kelty, 1982). There is a significant "back transfer" of metabolites from hydra to algae (Thorington and Margulis, 1981). If this "back transfer" is light driven, and if any of these metabolites are rate limiting with respect to regeneration, then a reasonable mechanism for light-induced inhibition of regeneration exists.

McAuley (1981) demonstrated that algal mitosis occurs under conditions where the host hydra cells are stimulated to divide. For example, he found that the mitotic index of regenerating peduncle cells is higher than non-regenerating controls. He suggests that the transient increase in algal cells relative to hydra cells, observed in regenerating peduncles, occurs simply because algal cells divide faster than host cells. Thus, when hydra tissue suffers a wound, both host and symbiont are stimulated to divide. Algal cells complete the mitotic cycle faster than host cells and a transient increase in their numbers within the hydra cells occurs.

In contrast, McAuley (1981) found no increase in the number of algae per cell in gastric tissue when hydra regenerated after a 1 day fast prior to decapitation. Similarly, we found no increase in the number of algae within gastric cells in hydra regenerating after a fast of 2 days, when hydra were taken from heavily fed stocks (Table V).

We could interpret our findings in terms of McAuley's hypothesis as follows: In well-fed hydra the number of cells in the gastric region is sufficient to regenerate missing parts solely by migration of cells. Therefore, since no hydra cell mitosis occurs prior to regeneration (Hicklin *et al.,* 1973; Webster, 1967; Corff and Burnett, 1969) algal cells are apparently not free to divide, and no transient increase in their numbers occurs.

After a fast of 10 to 15 days we found that the number of algae per gastric cell does increase, however, in regenerating hydra. We hypothesized that if an algal increase is observed, we would also find host cell mitosis elevated in regenerating animals. We did not. In Carolina, after a 9 day fast, the number of algae per gastric cell in regenerating animals is higher than non-regenerating controls after 48 hours of regeneration, but the mitotic index in these same animals is not higher than controls (Table VIII).

This result caused us to re-examine the proposal that a substance emanating from the hypostome can by itself directly regulate algal mitosis (Pardy *et al.,* 1976). Thus, when the hypostome is removed, an increase in algal cells relative to host cells could occur without a concurrent increase in host cell mitosis. It is not clear why this effect of decapitation on algal numbers is found only in fasted hydra, however. It is possible that one of the effects of fast is simply to alter the "timing" of algal mitosis with respect to mitosis of the host cell. Prolonged fast may selectively delay the animal cell's ability to respond to a mitotic stimulus. Under such conditions algal numbers could be elevated 48 hours after a wound without a concurrent increase in host cell mitosis.

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EFFECTS OF SALINITY ON FERTILIZATION SUCCESS IN TWO POPULATIONS OF *FUNDULUS HETEROCLITUS*

CATHERINE P. BUSH AND JUDITH S. WEIS[1]

Dept. of Zoology & Physiology, Rutgers University, Newark, N. J. 07102

ABSTRACT

A population of killifish (*Fundulus heteroclitus*) from northern NJ (Newark Bay and Piles Creek, salinity ~20‰) produces fertilizable ova only if stripped into reduced salinity water, while populations from estuarine areas of Long Island (Montauk and Southampton, salinity 20–25‰) produce viable eggs over a range of salinities (10–30‰). It was impossible to fertilize Piles Creek eggs in 30‰ even if sperm were already present in the water, but if eggs were transferred from 30‰ to 15‰ within one minute, successful fertilization was obtained. In full strength sea water (30‰) they became artificially activated and produced 90–100% noncleaving eggs, whereas Long Island eggs did not become artificially activated even after two hours in 30‰ salinity. The salinity to which sperm were initially exposed did not appear to affect their ability to fertilize ova from either population.

When Piles Creek eggs were stripped into 30‰ salinity in the absence or presence of sperm, SEM observations showed that the micropyles became blocked with extrusions seeming to originate from within the micropylar canal, and in some cases with cortical granules. This blockage very closely resembled the micropylar blockage seen in eggs after fertilization at 15‰ salinity. Ova stripped into 15‰ salinity without sperm generally had no micropylar blockage. This suggests that in the Piles Creek population, contact with full strength sea water rapidly initiates artificial activation, resulting in the blockage of the micropyle.

INTRODUCTION

Many investigators have addressed the issues of the fertilization process and artificial activation of the teleost ovum. Kagan (1935) noted that the fertilizable life of a *Fundulus heteroclitus* egg was 15–20 minutes. Beyond this time the percentage of successfully fertilized eggs became very small, because eggs became artificially activated, developing a perivitelline space after about 20 minutes in sea water. Kagan attributed the activation of the eggs to the sea water and concluded that the eggs which developed the perivitelline space (became activated) before insemination could not become fertilized. An artificially activated egg may form a one-cell stage, but it does not divide.

In teleost fertilization the sperm enters the ovum through a micropyle, which in *Fundulus* consists of a single opening in the chorion (Brummett and Dumont, 1981a) at the bottom of a slight depression. The micropyle apparatus consists of a relatively smooth sided funnel-shaped entrance, at the bottom of which is a 4–5 μ diameter pore, surrounded by slightly elevated lips. The micropylar canal diminishes in diameter (to 2–3 μ) as it approaches the plasma membrane of the ovum

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Abbreviations: NCE–noncleaving eggs, PC–Piles Creek, SH–Southampton.

[1] To whom reprint requests should be addressed.

(Kuchnow and Scott, 1977). Although the outer diameter of the micropylar funnel permits the entry of several sperm, the diameter of the inner opening allows only one sperm access to the egg (Brummett and Dumont, 1979).

Brummett and Dumont (1981a) in their ultrastructural studies on fertilization report that upon activation a "fertilization plug" is formed, sealing the inner opening of the micropyle and effectively preventing polyspermy. They speculated that artificially activated eggs might undergo the same micropylar blockage as eggs activated by fertilization. They found that cortical vesicle breakdown occurring upon activation could be somewhat explosive, liberating membrane fragments into the perivitelline fluid. Fibrous and particulate material as well as dense spheres were shown to be liberated from the wave of cortical vesicle breakdown. The breakdown of the cortical vesicles is a major structural change which might be the origin of the block to fertilization that is seen after artificial activation has occurred.

Fundulus heteroclitus is a common estuarine species, living in water ranging from fresh water to about 30‰ salinity. While studying pollution tolerance in embryos from Newark Bay and from Piles Creek in Linden, NJ, and from Montauk and Southampton, NY, it was discovered that when eggs of the Newark Bay or Piles Creek females (from ~20‰ salinity water) were stripped into 30‰ salinity water and mixed with sperm, the batches obtained would consist primarily of non-cleaving eggs (Weis *et al.*, 1981). Successful development would occur, however, if eggs were stripped into 15‰ salinity. Montauk or Southampton eggs (from sites of 20–25‰ salinity) could become fertilized and develop successfully at any salinity tested (10–30‰).

The present study is an attempt to analyze the unique response to sea water seen in the northern NJ ova in terms of timing, specific salinity, and morphological changes in the eggs.

MATERIALS AND METHODS

Mature, gravid *Fundulus heteroclitus*, collected at Piles Creek (PC) in Linden, NJ, or Southampton (SH), NY from May through July, were transported to the laboratory where they were separated by sex and maintained in aerated, filtered, diluted sea water (17–21‰ salinity) for several days. Ripe ova were obtained by stripping gravid females into finger bowls with sea water of a specific salinity. The sea water was prepared by dissolving artificial sea salts ("Rila Mix") in aged tap water and aerating the mixture overnight. All experiments were done at room temperature (~24°C). The ova were inseminated with milt stripped from males. Enough males were used in each experiment to assure an adequate supply of sperm. The experiments were designed so that the response of ova (from both populations) to different salinities could be determined.

(1) In the first experiment 602 ova from a single PC female were stripped and divided into two groups. Of these, 243 ova were inseminated in 30‰ salinity water with sperm from 3 PC males, and were maintained at this salinity for 5, 10, 15, 20, or 25 minutes, or until a one-cell stage had formed (approximately 60 minutes). They were then rinsed in 15‰ water and placed into 15‰ salinity water to develop. Development was considered to proceed normally if the blastodisc cleaved. In each group, the non-cleaving eggs (NCEs) were counted and the percentage of NCEs calculated. The reverse of this experiment was carried out simultaneously with the second group (359) of these eggs; *i.e.* the eggs were inseminated in and maintained at 15‰ salinity for corresponding time intervals before being transferred to 30‰ salinity. Similarly, 608 ova from a single SH female were divided into two groups,

and treated as above, except that they were inseminated with sperm from 3 SH males.

This experiment was repeated with 100 eggs from a PC female transferred from a 30‰ sperm suspension to 15‰, and another 100 eggs transferred from a 15‰ sperm suspension to 30‰ after 1, 2, 3, 4, and 5 minutes.

(2) To determine if 30‰ salinity activated the PC ova, eggs were stripped and were allowed to sit in 30‰ for up to three hours without sperm. Some were removed after 5 and 10 minutes, rinsed, and inseminated at 15‰ with milt from PC males. Other ova from the same females were maintained for up to three hours at 15‰ without sperm. Some of these were also removed at 5 and 10 minutes, rinsed, and inseminated with sperm from PC males in 30‰ salinity. A total of 201 ova from three females were used in these experiments.

(3) To determine the approximate salinity at which the PC ova would be fertilizable, ova were inseminated at 15‰, 20‰, 25‰, and 30‰ salinity. One such run was carried out using 77 ova stripped from a single PC female, inseminated with sperm from one PC male.

(4) To determine if exposure to either 30‰ or 15‰ salinity water would affect the ability of sperm to fertilize the ova, milt was stripped from PC males into a small volume of water of 15‰ or 30‰ salinity. Ova were stripped from PC females into separate finger bowls containing 30‰ and 15‰ salinity water respectively. The sperm suspension was then immediately added to the finger bowl containing the ova. (This would have changed the salinity slightly.) Sperm from SH males were also used in attempting to fertilize PC ova at 30‰ and 15‰ salinity. Ova from the SH population were used in the control runs. In a final experiment, milt was stripped from PC males into 30‰ salinity, and PC ova were stripped directly into the sperm suspension.

Specimens to be examined with the SEM were preserved in 0.07 *M* phosphate-buffered 4% glutaraldehyde solution for 2 and 3 days, after which they were dehydrated through a series of alcohols (30–100% EtOH) and acetone at 10-minute intervals. After dehydration, the specimens were critical point dried from CO_2 using a SAMDRI 960 (Tousimis) Critical Point Drier, and mounted on stubs with silver paint or double-stick tape and gold coating. The specimens were both inseminated and uninseminated eggs from PC females stripped into both 15‰ and 30‰ salinity. The durations of exposure of these ova to a given condition were 30 seconds, 2 minutes, and 3 minutes.

Forty ova were observed with a Scanning Electron Microscope (ISI IIIA) operated at 30 kV.

RESULTS

Population response to salinity

(1) The first experiment revealed that the effects of salinity on *Fundulus* ova occur within the first five minutes of contact with the medium. Table I shows that only the ova from the PC population are incapable of being successfully fertilized in full strength sea water. In 30‰ salinity water PC ova developed only up to the one-cell stage. Occasionally, however, these eggs did not form a perivitelline space or a one-cell stage. Regardless of whether an egg formed a perivitelline space, if it did not cleave it was included in the calculation of percentage NCEs. The SH ova, inseminated with SH sperm, were generally successfully fertilized (Table I) regardless of the salinity of the medium in these experiments.

TABLE 1

Results of experiments in which F. heteroclitus *ova are inseminated at one salinity and after a given interval of time are transferred to a second salinity*

Parent fish (male × female)	Fertilization salinity (FS) (‰)	Time spent in FS (minutes)	Final salinity (‰)	# of Ova	%NCE's
PC × PC	15	5	30	55	2
PC × PC	15	10	30	51	0
PC × PC	15	15	30	31	0
PC × PC	15	20	30	26	0
PC × PC	15	25	30	28	0
PC × PC	15	indefinite (~60)	15	52	0
PC × PC	30	5	15	103	95
PC × PC	30	10	15	77	97
PC × PC	30	15	15	40	97
PC × PC	30	20	15	34	95
PC × PC	30	25	15	50	86
PC × PC	30	indefinite (~60)	30	55	95
SH × SH	15	5	30	42	0
SH × SH	15	10	30	51	2
SH × SH	15	15	30	37	5
SH × SH	15	20	30	92	1
SH × SH	15	25	30	74	0
SH × SH	15	indefinite (~60)	15	47	4
SH × SH	30	5	15	73	10
SH × SH	30	10	15	57	2
SH × SH	30	15	15	41	0
SH × SH	30	20	15	40	0
SH × SH	30	25	15	61	2
SH × SH	30	indefinite (~60)	30	53	6

When determining if ova would cleave when removed from a 30‰ sperm suspension after a shorter period of time, it was observed that the PC ova were successfully fertilized if transferred from 30‰ to 15‰ within the first minute of exposure to 30‰ (Fig. 1a). It was also found that ova transferred from 15‰ to 30‰ within one minute produced a very high percent of NCEs (Fig. 1b). Figures 1a and 1b also include data from the longer exposures done initially.

(2) The second series, designed to discover whether the PC ova became activated and thus unfertilizable when exposed to 30‰ for 5 or 10 minutes before insemination at 15‰, resulted in 81% and 100% NCEs respectively. When fertilization was attempted at 30‰ after exposure to 15‰ for 5 and 10 minutes, 100% and 90% NCEs were obtained. However, if inseminated in the 15‰ salinity after comparable periods, fertilization was successful with almost no NCEs (3%). Such data are consistent with previous results.

(3) The investigations of what critical salinity prevented fertilization revealed that successful fertilization could occur at 15‰, 20‰, and 25‰ salinity (Fig. 2) although at 25‰ some reduced success was evident. These data are taken from eggs of one female, however, and there is probably some variability within the population.

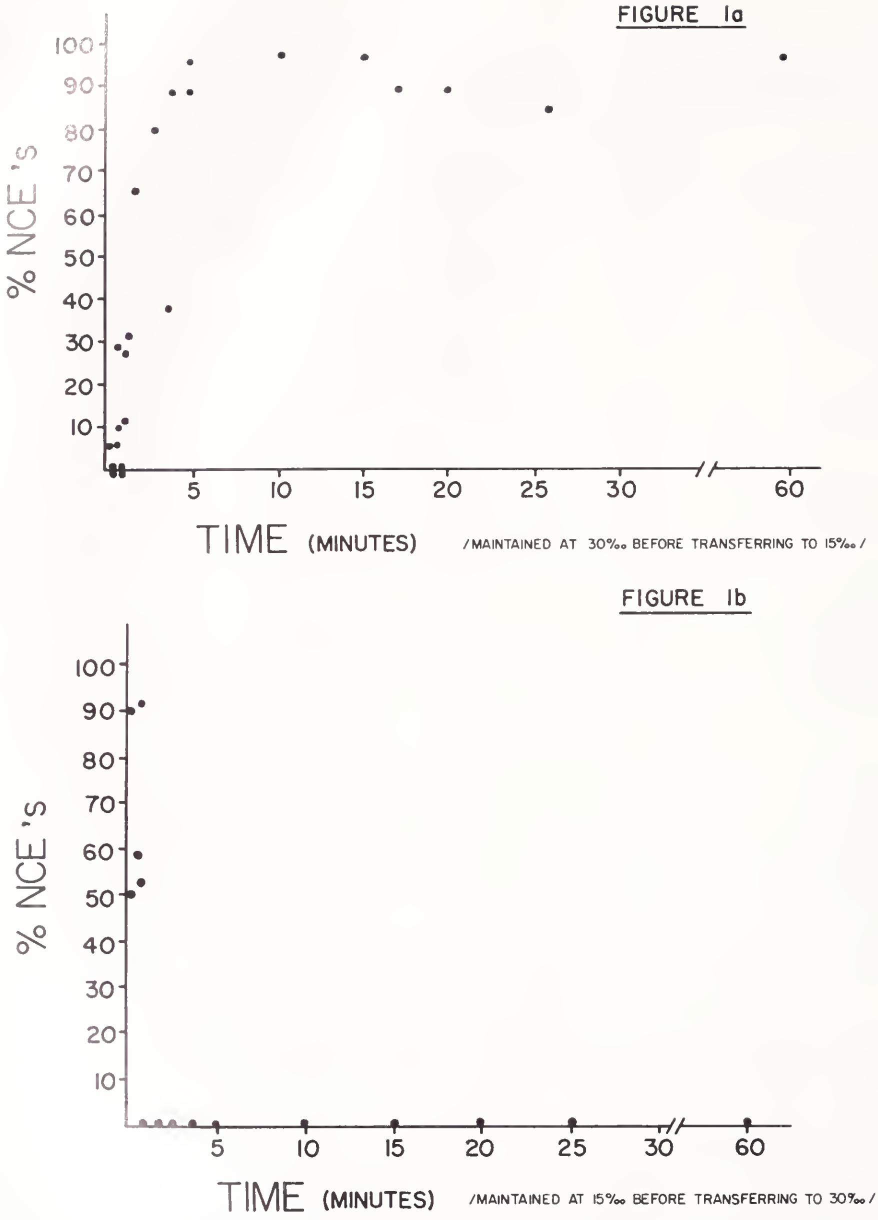


FIGURE 1. a) Percentage of noncleaving eggs (%NCE's) obtained if a batch is allowed to remain in 30‰ salinity with sperm for a given time interval before being transferred to 15‰ salinity. Each point represents a single batch of eggs (20–40 ova) observed. b) %NCE's obtained if a batch is allowed to remain in 15‰ salinity with sperm for a given time interval before being transferred to 30‰ salinity.

(4) In the experiments determining if exposure of sperm to different salinities would affect their ability to fertilize PC ova, it was found that the exposure of sperm of either population to various salinities did not affect their ability to successfully fertilize ova. An average of 73% NCEs was obtained from 4 replicate experiments

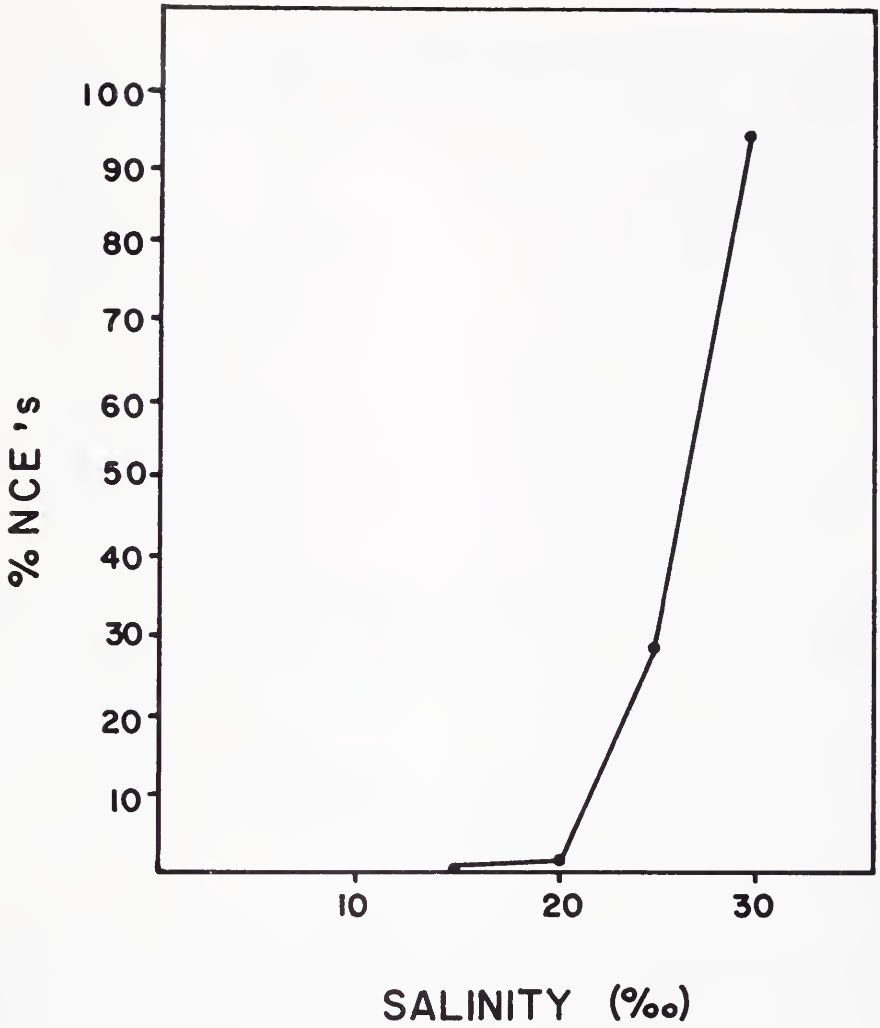


FIGURE 2. %NCE's as a function of the salinity.

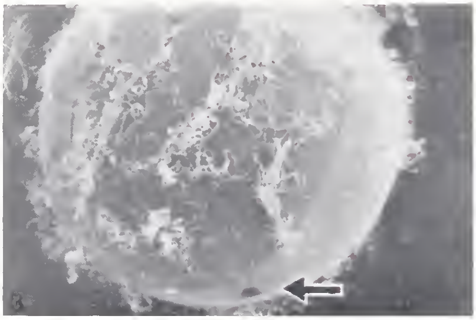
in which PC sperm were exposed to 15‰ for one minute and then used to inseminate ova at 30‰ salinity. When this same procedure was performed with a reversal of the salinities (sperm in 30‰, eggs in 15‰) most of the eggs cleaved successfully, with the NCEs averaging 7% in six replicates. When sperm from SH males was used to inseminate PC ova at either salinity, the results were comparable. When eggs were at 30‰ and sperm at 15‰ salinity the NCEs averaged 65% in four replicates, and when eggs were at 15‰ and sperm at 30‰ salinity the NCEs averaged 5% in four replicates. SH eggs in 15‰ or 30‰ salinity inseminated with sperm from either PC or SH males all averaged less than 10% NCEs. In the final experiment, in which PC ova were stripped directly into a sperm suspension in 30‰ salinity, 88% NCEs were obtained. Ova from the same female were sucessfully fertilized (7% NCEs) using the above technique in 15‰ salinity sea water.

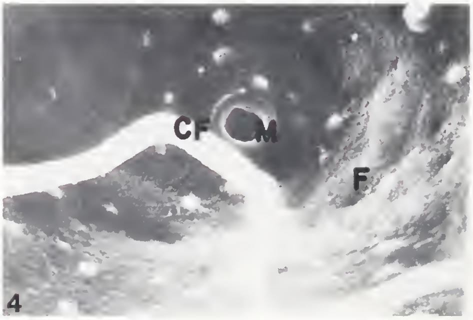
SEM observations of ova

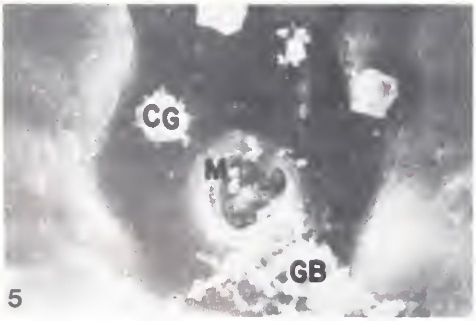
SEM views of the ova at low magnification (Fig. 3) show the micropyle as a conical depression in the surface. Detailed observations on micropylar regions of 40 ova which were exposed to different salinities for varying intervals of time showed the following results: Exposure of ova to 15‰ salinity without sperm for up to three minutes resulted in 60% of the micropyles remaining open. Figure 4 depicts one such open micropylar canal. There is no evidence of extrusions of any kind that would lead to blockade of the canal and, thus, exclusion of sperm.

When PC ova were inseminated in 15‰ salinity, 92% of the micropyles were blocked by what seemed to be extrusions from within the micropylar canal. Figure 5 illustrates a type of obstruction that is particulate in nature. Figure 6 illustrates what seems to be the release of cortical granules (2.75 μ in diameter) through the opening of the micropyle. All SEM pictures in this set are of ova removed after 30 seconds of exposure to a 15‰ salinity solution containing sperm.

In 90% of the ova observed under conditions of 30‰ and salinity and the absence of sperm, particulate, fibrillar, or spherical blocks formed around the micropyle within 30 seconds of exposure (Fig. 7). Figure 7 also illustrates an interesting ob-







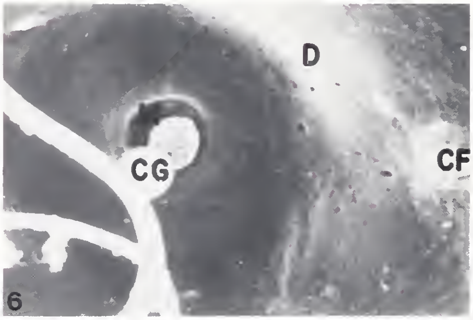


FIGURE 3. Low magnification SEM of an ovum fixed immediately after shedding. Arrow points to the location of the micropyle. Long chorionic fibers are present on the surface of the ovum. (Magnification ×50)

FIGURE 4. A SEM view of the micropyle (M) on the outer surface of the ovum fixed after 30 s in 15‰ salinity without sperm. No chorionic fibers (CF) are present on the inside of the funnel (F) leading to the micropyle. The elevated lip is visible in this picture. (Magnification ×2000)

FIGURE 5. A micropyle (M) obviously blocked by cortical granules (CG) and granular extrusions (GB); ovum inseminated at 15‰ salinity and fixed at 30 s after insemination. (Magnification ×4000)

FIGURE 6. Cortical granules (CG) being extruded from within the micropyle; ovum inseminated in 15‰ salinity and fixed 30 s after insemination. Chorionic fibers (CF) are visible at the edges of the depression (D) leading to the micropyle. (Magnification ×4000)

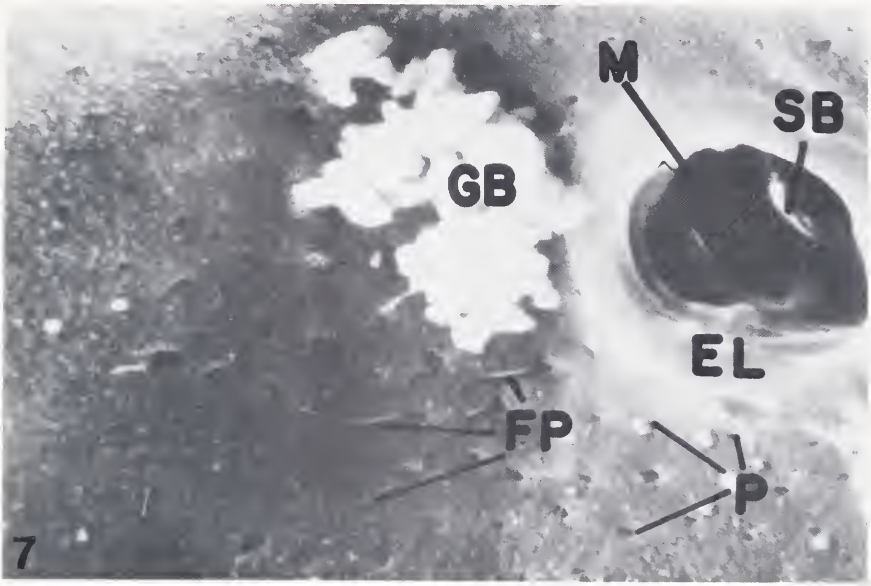


FIGURE 7. An elevated lip (EL) is visible surrounding the micropyle (M). There are pores (P) around the micropyle with fibrillar particles (FP) extending out of them. Ovum was fixed after 2 min exposure to 30‰ salinity with no sperm. Granular extrusions (GB) and a spherical block (SB) are visible. (Magnification ×5000)

servation. Pores are present around the micropyle, and fibrils can be seen extending out of them. Such pores were observed to be present around all micropyles that could be closely examined, but the fibrils were not always present in these pores. Our data reveal no consistent pattern of presence or absence of these fibrils.

Finally, ova subjected to insemination at 30‰ salinity revealed blockage of the micropyles in 100% of the specimens observed. Blockage was very similar to that observed under all the other conditions: the blocks were either spherical, fibrillar, or granular. Figure 8 illustrates blockage at 30‰ salinity in the presence of sperm. There is no way to determine if this egg was artificially activated or successfully fertilized, although, judging from experimental data, most such eggs were artificially activated by the water.

DISCUSSION

The data reveal a striking difference in the ability of ova of the two populations to become fertilized at 30‰ salinity and no difference if fertilization was attempted at 15‰ salinity. The PC population could not produce viable gametes at 30‰, even if eggs were stripped directly into a sperm suspension. Only if they were changed from 30‰ to 15‰ salinity within one minute would successful development occur.

Rao (1974) studied the influence of salinity on fertilization of *Fundulus parvipinnis* inhabiting the bays, lagoons, and fresh water areas along the southern California coast. He found successful fertilization to occur within the salinity range of 5‰ to 33‰, or those salinities that constituted the natural conditions for this species.

F. heteroclitus lives in fresh water, sea water (30‰–34‰) and all intermediate salinities (Feldmeth and Waggoner, 1972). Therefore, one might expect that they should be able to produce viable offspring in a wide range of salinities. The SH

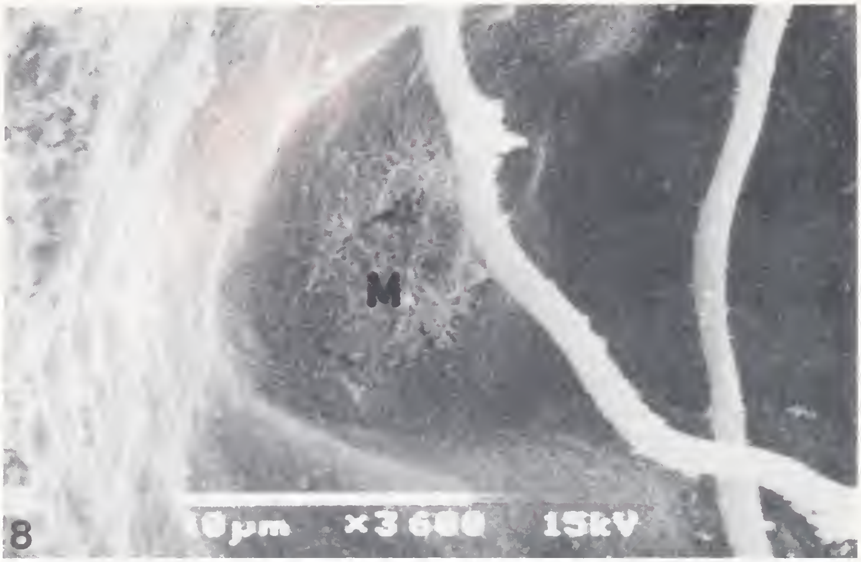


FIGURE 8. An ovum fixed 3 min after insemination in 30‰ salinity. Total blockage of the micropyle (M) has occurred. (Magnification ×3600)

population lives in a less than full strength sea water habitat (20–25‰), and produces viable offspring at varying salinities. The PC population (~20‰) however, could not produce viable offspring at 30‰ salinity.

Holliday (1971) reported that crossfertilization between the races of fish species results in responses characteristic of the female parent. In our observations, cross-fertilization between the two populations similarly resulted in a response to salinity that was characteristic of the origin of the female. The ability of sperm to fertilize ova was not affected by the salinities tested.

Brummett and Dumont (1979) found that activation of the *Fundulus heteroclitus* ovum by fertilization is followed by formation of a block to polyspermy in the form of a "fertilization plug" consisting of numerous globules of various sizes. It would follow that if a type of "plug" were formed upon artificial activation, it would also prevent access of sperm to the ovum and would render such ova unfertilizable.

From SEM views of the micropyles it is clear that upon artificial activation the micropyle becomes blocked with globular, particulate, or fibrillar particles. There seems to be no definite correlation between the type of block and whether the egg has been artificially activated. We are unsure why 40% of the eggs which were uninseminated in 15‰ had micropylar blocks when experimental data indicate that they are almost all fertilizable. It is possible that the fixative could have activated some of the eggs.

Brummett and Dumont (1979, 1981a) have shown that cortical vesicle breakdown and extrusion of the contents begins to occur one second after insemination. They examined the contents of these vesicles, finding large (9 μ) and small (1.25 μ) granules, and still smaller globular and particulate matter. Dumont and Brummett (1980) also noted the pores around the micropyle but did not think that they perforated the chorion. Nevertheless, it may be possible that the explosive extrusion of granules from the cortical vesicles sends material out through the pores (which were found around all observed micropyles) and also thereby blocks the micropyles.

It is also possible, if the pores do not perforate the chorion, that the fibrils we observed were actually the inner lining of the pores turned inside out by the explosive activation process. It is likely that activation of PC ova by sea water involves the same cortical vesicle breakdown as activation by sperm.

There is evidence that the high salinity must interfere with sperm-egg interaction prior to cortical vesicle breakdown. The reasons for this are that the effect of 30‰ salinity has not progressed far enough to prevent fertilization if the inseminated eggs are transferred to 15‰ within one minute. Yet, 88% of the eggs failed to fertilize when stripped directly into a sperm suspension (in which sperm could certainly get to the eggs within the first minute). Thus, fertilization fails to occur in 30‰ salinity even when the sperm could have immediate access to the eggs. This strongly suggests that high salt somehow interferes with the direct sperm/egg interactions which lead to fertilization. Another possibility is that PC eggs might, upon contact with 30‰ water, secrete some substance which blocks sperm/egg interactions but which is dissolved by 15‰ salinity. No evidence for such a coating has been seen, however.

In considering the responses of the two populations to salinity, we should note the environments to which they have adapted. The salinities in which the parent fish live and have developed vary slightly between the two populations. However, Kinne (1962) suggests that from the evidence available, the induced differences due to spawning in varied salinity are not transmitted to the next generation. From these studies it would seem that killifish living in ~20‰ salinity in PC and fish living in ~25‰ salinity in SH should produce ova equally tolerant to varying salinities. Acclimation of the female fish to one or another salinity should not be responsible for the behavior of the ova.

Southampton is a relatively pristine area, while there is thermal, heavy metal, and oil pollution at Piles Creek, a tributary of the Arthur Kill. Gametes of fishes are expected to have only a short period of independent life, so the period of tolerance in nature would not have to exceed that required for successful fertilization. In nature, sperm and ova are codeposited and therefore the exposure to the medium, prior to fertilization, should be minimal. Given the conditions to which the PC population is exposed, *i.e.* presence of multiple pollution, it might be beneficial to the population if the ova were fertilizable for the shortest period of time possible, thus ensuring the lowest uptake of toxicants and possibly more successful embryonic development. The chorion becomes more impermeable over time (Tay and Garside, 1975) and thus more resistant to uptake of pollutants.

Weis *et al.* (1981) found that the PC embryos were more resistant to teratological effects of methylmercury than were embryos from Montauk or Southampton, which showed much greater variation in susceptibility, ranging from highly tolerant to highly susceptible. Perhaps the PC population has adapted narrowly to the specific conditions of its habitat and cannot tolerate changes in its environment. This might account for the inability of PC ova to fertilize in 30‰ (an "unnatural" environment). Furthermore, Renna (1982) noted that the PC adults had a much higher mortality in artificial sea water than the SH adults, which may also indicate a reduced ability to adjust. The PC fish seem to have adapted narrowly to the conditions of Piles Creek and to have lost some euryplasticity in the process.

Another possibility that should be considered is that the PC adults are under stress living in a polluted environment. Renna (1982) found that survival and fin regeneration rate of PC adults was much lower than SH adults in both clean water and methylmercury-contaminated water. This could indicate a weakened population of fish. Weakened adults might produce weakened gametes that might not be as adaptable as their counterparts from clean environments. The lack of adaptability is true for ova but not for sperm in this case, since the ability of sperm to fertilize ova was not affected by salinity.

On the other hand, the differences between the ova response to salinity in the two populations might not be pollution-related at all, but may be a component of major differences in reproductive biology within this species. A variety of differences have been found which vary clinally along a north-south gradient. Southern populations of *F. heteroclitus* tend to produce eggs with more numerous, shorter chorionic fibrils (Brummett and Dumont, 1981b), more numerous oil droplets (Morin and Able, 1980), and to have their reproduction more closely tied to a lunar cycle than do more northern "races" (Wallace and Selman, 1981). Southern populations have been observed to deposit their eggs in *Modiolus* shells (Able and Castagna, 1975), a reproductive behavior not observed in more northern fish. Gene frequency differences have been noted (Place and Powers, 1978) which have been correlated with egg hatching times (DiMichele and Powers, 1982). The populations studied here are both from the intergrade zone, but in terms of oil droplet counts, chorionic fibrils, isozymes, and reproductive periodicity, the PC population tends to be closer to the southern type and the Long Island populations to the northern type (Heber, 1981). However, the fibers which we observed extending out of the pores around the micropyle of PC eggs were also observed by Brummett (personal communication) in eggs from Woods Hole, but not from South Carolina. Given the many differences in reproductive biology, it would therefore not be surprising if egg responses to salinity might also vary geographically.

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ENHANCED REDUCTION OF T4D AND T7 COLIPHAGE TITERS FROM *BIOMPHALARIA GLABRATA* (MOLLUSCA) HEMOLYMPH INDUCED BY PREVIOUS HOMOLOGOUS CHALLENGE

THOMAS C. CHENG, KEITH H. HOWLAND, AND JOHN T. SULLIVAN

Marine Biomedical Research Program and Department of Anatomy (Cell Biology), Medical University of South Carolina, Charleston, SC 29412

ABSTRACT

The hemolymph of *Biomphalaria glabrata* contains a soluble factor(s) that neutralizes the coliphages T4D and T7 *in vitro.* This neutralization is not enhanced by prior injection of the snail with the homologous or heterologous phage. *In vivo* titers of injected T4D and T7 decrease as a function of time postinjection (PI), with a nearly 1000-fold reduction occurring by 144 h PI. When snails are reinjected at 96 h PI with homologous phage, the *in vivo* titers of infective T4D and T7 decrease even more rapidly. Specifically, T7 titers at 24, 48, and 72 h post-reinjection (PR) are significantly lower than those at the same time periods PI, *i.e.,* following a single injection. Similarly, T4D titers at 24, 48, 72, and 96 h PR are significantly lower than at 24, 48, 72, and 96 h PI. It is concluded that the reduction of phage titer in the hemolymph of injected *B. glabrata* is due in part to neutralization by some naturally occurring factor(s) and also to an active, probably cellular, mechanism that is enhanced by previous homologous challenge. The specificity of this enhancement is not yet known; however, prior sham or Tris diluent injections do not affect the titers of a subsequent T4D or T7 injection.

INTRODUCTION

Among vertebrates, specificity and anamnesis (memory) are two primary features of the humoral and cellular immune responses. It is now generally accepted that anamnesis reflects a specific secondary response that is both more rapid and of greater magnitude than the primary response to antigenic challenge. Invertebrates, although capable of discriminating between self and nonself (see Chorney and Cheng, 1980; Hildemann *et al.,* 1980b; Lackie, 1980; and Warr, 1981 for reviews), do not possess vertebrate-type immunoglobulins. Thus, inducible memory would most likely be revealed as selectively altered reactivity upon secondary contact in some form of cell-mediated immunity (Hildemann *et al.,* 1980b). Recent data on allograft rejection in invertebrates (see Hildemann *et al.,* 1980b for review) suggests that at least in the sponges, coelenterates, echinoderms, and possibly annelids, invertebrates are capable of a specific immune response with at least short-term memory.

Although there is some evidence that bivalve molluscs, specifically oysters (*Crassostrea virginica*), can be induced to engage in enhanced clearance of T2 coliphage resulting from second challenge (Acton and Evans, 1968), the experimental protocol employed, involving half-shell preparations, is open to question. Consequently, reported herein are the results of studies on the reduction of circulating, infective coliphages from the hemolymph of *Biomphalaria glabrata,* a gastropod mollusc, which did not require removal of an entire valve. Evidence is presented that these

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molluscs possess (1) some naturally occurring phage-neutralizing factor(s) in their serum; and (2) a second, possibly cellular, mechanism that is enhanced by prior homologous challenge.

MATERIALS AND METHODS

Snails

The specimens of *B. glabrata* used in this study were of the so-called NIH albino strain (Newton, 1955). All of the snails measured from 7–15 mm in shell diameter. They were maintained in 3-liter glass aquaria containing deionized water fortified with Nolan and Carriker's (1946) salt solution at a concentration of 1.58 ml/liter and fed romaine lettuce *ad libitum.*

Bacteriophages

The bacteriophages employed were the coliphages T4D and T7. They were originally obtained from Dr. L. Chao of the Department of Biochemistry, Medical University of South Carolina. The phages were harvested from *Escherichia coli* strain B (ATCC 11303). The titers of the phage suspensions used were determined weekly by the standard plaque assay method (Douglas, 1975). For injection experiments, titers of T4D and T7 were adjusted with sterile Tris diluent (1 ml of 1 *M* $MgCl_2$, 21.5 ml of 4 *M* NaCl, 10 ml of 1 *M* Tris-HCl, pH 7.5, 1 ml of 1% gelatin, and 4 ml of 0.025 *M* L-tryptophan/liter of water) to provide a suspension containing 2×10^8 plaque forming units (PFUs)/ml.

Injection procedure

The injection of snails, except for those sham injected, was accomplished by a modification of the method of Sullivan and Cheng (1976). In brief, a small aperture was made on the left side of the shell overlaying the anterior region of the digestive gland where the postintestine and midintestine lie parallel to each other. A microcapillary tube, which had been drawn to a fine point, containing 2 μl of either the phage suspension or the control solution, was inserted through the aperture into the sinus situated immediately anterior to the digestive gland. The solutions were introduced slowly as a result of pressure from a mercury column. After the microcapillary was retracted, each snail was permitted to air dry for 5 min prior to being placed in a 400-ml beaker containing deionized water fortified with Nolan and Carriker's (1946) solution. A maximum of six snails was placed in each beaker, the water was changed daily, and the animals were fed lettuce *ad libitum.*

In vitro *neutralization assay*

The ability of snail serum to neutralize bacteriophage was assayed. Fifty snails were divided into five equal groups: (1) The first group was left untampered. (2) The second group received an injection of 2 μl of the T4D suspension (2×10^8 PFUs/ml). (3) The third group received an injection of 2 μl of the T7 suspension (2×10^8 PFUs/ml). (4) The fourth group received an injection of 2 μl of sterile Tris diluent. (5) Finally, the fifth group received a sham injection (*i.e.,* inserting the needle into the hemocoel for 30 s). Snails were bled 24 h post-challenge by making a small hole in the shell directly over the heart and inserting a sterile capillary tube. At least 30 μl of hemolymph was collected from each snail. The samples were subsequently placed into individual 400-μl microcentrifuge tubes and the cells were separated from sera by centrifugation at 1000 g for 10 min in a Beckman Model J2-21 refrigerated centrifuge. The sera were transferred to new 400-μl microcentrifuge tubes and kept at 4°C until assayed.

A 10 μl sample of both undiluted and diluted (1:100 in Tris diluent) serum from each snail from the five groups was combined with either 10 μl of a suspension of 2×10^4 PFUs of T4D phage/ml of Tris diluent, 10 μl of a suspension of 2×10^4 PFUs of T7 phage/ml of Tris diluent, or 10 μl of Tris diluent and incubated for 2 h at 25°C. Following incubation, the samples were assayed for bacteriophage by a modification of the standard plaque assay method (Douglas, 1975). Specifically, the reaction mixture, 20 μl in total, was used instead of 100 μl of serum. The plates were incubated at 25°C and the plaques were counted and recorded as PFUs/ml of serum. The assay was repeated once using a second group of 50 snails.

In vivo *assay*

The 664 snails employed in each experiment were divided into nine groups: (1) The 80 snails comprising the first group were each injected with 2 μl of the T4D suspension (2×10^8 PFUs/ml). (2) The 80 snails comprising the second group were each injected with 2 μl of the T7 suspension (2×10^8 PFUs/ml). (3) Each of the members of the third group, consisting of 80 snails, received two injections, each consisting of 2 μl of the T4D suspension, 96 h apart. (4) Each of the members of the fourth group, consisting of 80 snails, received two injections, each consisting of 2 μl of the T7 suspension, 96 h apart. (5) The 80 snails comprising the fifth group each received two injections 96 h apart; the first injection consisted of 2 μl of Tris diluent and the second injection consisted of 2 μl of the T4D suspension. (6) The 80 snails comprising the sixth group each also received two injections 96 h apart; the first injection consisted of 2 μl of Tris diluent while the second injection consisted of 2 μl of the T7 suspension. (7) Each of the 80 snails comprising the seventh group was initially sham injected followed 96 h later by an injection of 2 μl of the T4D suspension. (8) Each of the 80 snails comprising the eighth group was similarly sham injected 96 h prior to receiving an injection of 2 μl of the T7 suspension. (9) Finally, each of the 24 snails comprising the ninth group was left untampered. This injection experiment was replicated once, using an additional 664 snails.

The time interval of 96 h between injections in Groups 3 through 8 was chosen based upon the results of the single injections. The levels of viable phage remaining in the hemolymph of snails given a single injection of either T4D or T7 were relatively low at 96 h PI (approximately 1000 PFUs/ml).

Collection of hemolymph

At 6, 12, 24, 48, 72, 96, 120, and 144 h after either a single injection of coliphage or after the second injection of the paired injection series, individual hemolymph samples were collected from each of 10 snails from Groups 1 through 8 as described above. The cells were separated from sera as described above and the sera were stored for no more than 12 h at 4°C before being assayed.

Plaque assays

The serum samples were assayed from bacteriophage by employing a modification of the standard plaque assay method (Douglas, 1975). Specifically, we had to use ten times less serum (*i.e.,* 10 μl) because of the small amount of hemolymph obtainable from each snail. The plates were incubated at 25°C and the plaques were counted and recorded as PFUs/ml of serum. If confluent plaques occurred, a serial ten-fold dilution series was prepared and assayed for bacteriophage.

Statistical analysis

The Student's two-tailed t test was employed to determine whether there were statistically significant differences between the mean serum titers in the control groups (Groups 1, 2, 5, 6, 7, 8, and 9) and experimentally treated groups (Groups 3 and 4) of snails in the *in vivo* assays and between the mean serum titers in the control group (untampered) and experimentally treated groups of snails in the *in vitro* neutralization assay.

Statistical analyses were performed on untransformed data. However, the data are presented graphically as the logarithm of the actual mean titers due to the wide range of titers encountered over the course of the experiments.

RESULTS

The *in vitro* and *in vivo* experiments, as explained, were repeated once, using a second group of snails for each. Since the results of all replicates were not significantly different, data were pooled, and for purposes of computing mean titer values were considered as a single large experiment.

Neutralization test

Table I presents the combined mean titers obtained from two replicate experiments in which sera from the five groups of snails were incubated with either 2×10^4 PFUs of T4D or T7/ml of Tris diluent *in vitro* for 2 h at 25°C. Statistically significant differences were observed in the following instances. Specifically, sera from snails previously challenged with either T7 or with Tris diluent and then incubated with T4D *in vitro* had a significantly higher ($P < 0.05$) mean titer than did serum from untampered snails incubated with T4D. Also, sera from snails previously challenged with either T4D or with Tris diluent and then incubated with T7 *in vitro* had a significantly higher ($P < 0.05$) mean titer than did serum from

TABLE I

Pooled results of two neutralization assays of serum from Biomphalaria glabrata *combined with the bacteriophages T4D and T7* in vitro

Serum sample	Combined with T4D (2×10^4 PFUs/ml) (n = 20 snails)	Combined with T7 (2×10^4 PFUs/ml) (n = 20 snails)
Untampered	2,480.0 ± 561.577	2,655.0 ± 551.052
T4D Injected	7,480.0 ± 8,126.150	9,280.0 ± 4,878,373[B]
T7 Injected	4,185.0 ± 1,729.093[A]	2,550.0 ± 1,417.373[D]
Tris Diluent Injected	3,570.0 ± 564.847[A,C]	3,395.0 ± 955.579[B,D]
Sham Injected	2,570.0 ± 863.347[C]	2,975.0 ± 1,096.826[D]

Entries represent the combined mean numbers of PFUs/ml of serum ± the standard deviations.

[A] Significantly greater ($P < 0.05$) than untampered snail serum combined with T4D.

[B] Significantly greater ($P < 0.05$) than untampered snail serum combined with T7.

[C] Significantly less ($P < 0.05$) than T4D injected snail serum combined with T4D.

[D] Significantly less ($P < 0.05$) than T4D injected snail serum combined with T7.

untampered snails incubated with T7. In addition, the mean titer from snails challenged previously with T4D and incubated with T4D *in vitro* was significantly higher ($P < 0.05$) than that obtained for sera from snails previously challenged with either Tris diluent or with sham injections and incubated with T4D *in vitro*. Furthermore, the mean titer from snails previously challenged with T4D and incubated with T7 *in vitro* was significantly higher ($P < 0.05$) than that obtained for sera from snails previously challenged with T7, with Tris diluent, and with sham injections and incubated with T7. No other significant differences were observed among the experimental groups.

Incubation of each serum sample from the five groups of snails with Tris diluent alone revealed that there were no phage present in the sera of any of the noninjected, the Tris diluent injected, and the sham injected snails. There were some phage remaining in the sera of all of the T4D injected and T7 injected snails. This background value for each snail was subtracted from the value obtained upon incubation of its serum with either T4D or T7 *in vitro* to obtain the mean titers shown in Table I.

Dilution of serum (1:100) with Tris diluent caused a loss in the ability of the serum to neutralize the phages. When diluted serum was incubated with T4D or T7 *in vitro* and then was assayed for phage, confluent plaques were obtained for all snails in all five groups.

In vivo *assay*

Figure 1 presents the combined mean serum titers from replicate experiments expressed as the logarithm_{10} of the mean numbers of PFUs/ml of serum, as a function of either time after a single injection or time after the second of two injections, ascertained when *B. glabrata* were challenged with (1) a single injection of coliphage T4D, (2) a double injection of T4D administered 96 h apart, (3) an injection of sterile Tris diluent followed 96 h later by an injection of T4D, and (4) a sham injection followed 96 h later by an injection of T4D.

Statistical analyses revealed that at 6 and 12 h after the second of two injections of T4D, the mean serum titers were not significantly different from those obtained for singly injected snails or those obtained for snails that had been challenged with either an initial injection of Tris diluent or a sham injection followed 96 h later by injection of T4D. At 24, 48, 72, and 96 h after injection with the second of the two challenges with T4D, the mean serum titers for these snails were significantly less ($P < 0.05$) than those obtained from singly injected snails, snails that had been injected with Tris diluent followed 96 h later by an injection of T4D, and snails receiving a sham injection followed 96 h later by an injection of T4D. At 120 and 144 h after the second of two injections of T4D, there were no significant differences between the mean serum titers.

Figure 2 presents similar data obtained from snails subjected to the same series of injections as above, except that coliphage T7 was substituted for T4D.

Statistical analyses revealed that at 6 and 12 h after the second of two injections of T7, the mean serum titers were not significantly different from those obtained for singly injected snails and those obtained for snails that had been challenged with either Tris diluent or a sham injection initially followed 96 h later by an injection of T7. At 24, 48, and 72 h after injection with the second of the two challenges with T7, the mean serum titers for these snails were significantly less ($P < 0.05$) than those obtained for singly injected snails, snails that had been injected with Tris diluent followed 96 h later by an injection of T7, and snails receiving a sham

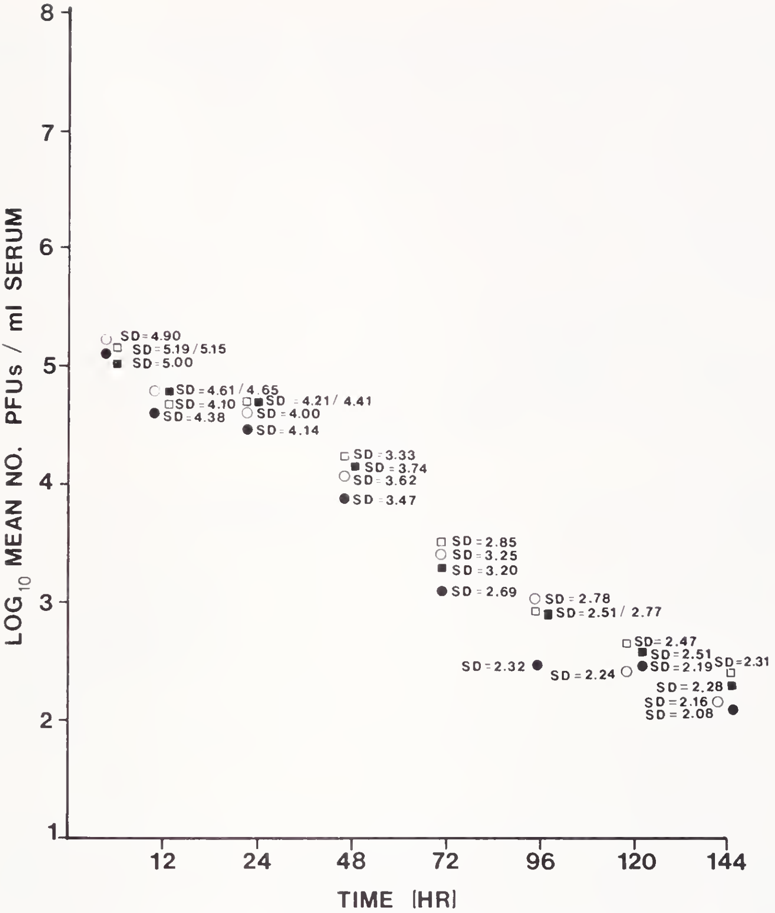

FIGURE 1. Logarithm$_{10}$ of the combined mean number of PFUs of T4D phage/ml of serum obtained from *Biomphalaria glabrata* given a single injection of T4D (○), a pair of injections of T4D (●) given 96 h apart, an injection of Tris diluent followed 96 h later by an injection of T4D (□), and a sham injection followed 96 h later by an injection of T4D (■). Each point represents the logarithm$_{10}$ of the mean number of PFUs of T4D/ml of serum from 18–20 snails, pooled from the two separate experiments. Standard deviations (SD) shown equal the logarithm$_{10}$ of the standard deviations.

injection followed 96 h later by an injection of T7. Furthermore, at 96 h after the second of two injections of T7, the mean serum titer was significantly lower ($P < 0.05$) than that for snails receiving a sham injection followed 96 h later by an injection of T7, but it was not significantly different from those for singly injected snails and snails initially challenged with Tris diluent followed 96 h later by an injection of T7. At 120 and 144 h post challenge with the second injection of T7, there were no significant differences observed between the mean serum titers.

Some mortality occurred among eight of the nine groups of snails. Specifically, the mortality rates among members of Groups 1 through 8 were as follows: Group 1, 5 out of 160 (3.1%); Group 2, 4 out of 160 (2.5%); Group 3, 3 out of 160 (1.9%); Group 4, 4 out of 160 (2.5%); Group 5, 4 out of 160 (2.5%); Group 6, 9 out of 160 (5.6%); Group 7, 4 out of 160 (2.5%); and Group 8, 4 out of 160 (2.5%).

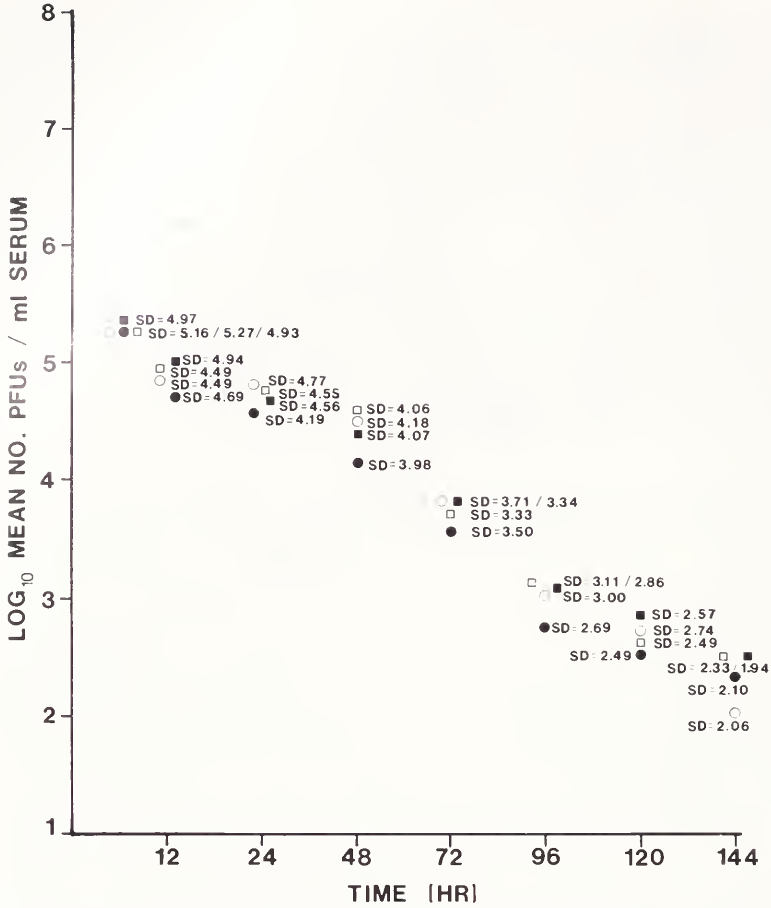

FIGURE 2. Logarithm$_{10}$ of the combined mean number of PFUs of T7 phage/ml of serum obtained from *Biomphalaria glabrata* given a single injection of T7 (○), a pair of injections of T7 (●) given 96 h apart, an injection of Tris diluent followed 96 h later by an injection of T7 (□), and a sham injection followed 96 h later by an injection of T7 (■). Each point represents the logarithm$_{10}$ of the mean number of PFUs of T7/ml of serum from 18–20 snails, pooled from the two separate experiments. Standard deviations (SD) shown equal the logarithm$_{10}$ of the standard deviations.

Quantitative PFU assays were also carried out on the sera of 48 untampered snails (Group 9). No coliphages were detected.

DISCUSSION

There are several reports which suggest that invertebrates are capable of mounting an enhanced response to post-primary challenge(s). As examples, Evans *et al.* (1969) have reported that when the spiny lobster, *Panulirus argus,* is challenged with a Gram-negative bacillus identified as EMB-1, a hemolymph bactericidin is synthesized. More important, they reported that the bactericidin titer is increased as a result of secondary and tertiary challenge with EMB-1. More recently, Karp and Rheins (1980) have demonstrated a humoral response induced in the American cockroach, *Periplaneta americana,* to the soluble protein complexes of honey bee (*Apis mellifera*) toxin and western cottonmouth moccasin (*Agkistrodon piscivorus*) venom. The response was specific for the original immunizing protein and a second challenge with the same antigen induced a classic secondary response in primed animals, thus suggesting the existence of immune memory. Also, Hildemann *et al.* (1977) have reported that there is development of a specific memory in the scleractinian coral *Montipora verrucosa* as a result of second-set grafts, and Hildemann *et al.* (1980a) have reported accelerated reactivity to second-set grafts in the sponge *Callyspongia diffusa.* Similarly, Langlet and Bierne (1977) have reported that there is enhanced rejection of second-set grafts among nemertines of the genus *Lineus.* The accelerated rejection of second- and third-set allografts has also been reported for the sea star *Dermasterias imbricata* (Karp and Hildemann, 1976).

Based upon the criterion of enhanced graft rejection, Cooper (1968, 1969, 1970) has promoted the idea that immunologic memory occurs among annelids; however, his results and interpretations have been challenged by Dales (1978) and Parry (1978).

Among molluscs, the enhanced clearance of T2 coliphage from the oyster *C. virginica* following second challenge (Acton and Evans, 1968) has been mentioned. Criticism of this study may be leveled at the fact that half-shell preparations were employed. Since bivalves, like gastropods, have an open circulatory system, the removal of one of the valves, which involves cutting the adductor muscle, could result in a nonphysiological state involving significant hemorrhage. Consequently, the results of that study remain open to question.

Lie and Heyneman (1975) and Lie *et al.* (1975) have reported what could be interpreted to be an enhanced reaction in a gastropod mollusc resulting from prior challenge. Specifically, they have found that when naturally resistant juvenile *B. glabrata* are challenged with normal miracidia of the digenean *Echinostoma lindoense* (Lie and Heyneman, 1975), or when susceptible snails are challenged with irradiated miracidia (Lie *et al.,* 1975), the parasites migrate to the heart where they become encapsulated by hemocytes and are destroyed. If such snails are subsequently challenged with normal miracidia, the parasites are destroyed rapidly by hemocytes near the penetration site in the head-foot. These observations suggest that initial challenge enhances the cidal effects of the hemocytic response to the second challenge with the parasite.

It now appears that *B. glabrata* is capable of recognizing certain viruses and eliminating them from their hemolymph. Specifically, these molluscs, as observed previously in crayfish (Sloan *et al.,* 1975) and blue crabs (McCumber and Clem, 1977; McCumber *et al.,* 1979), have naturally occurring levels of a soluble phage neutralization factor(s) in their serum, as shown by neutralization of T4D and T7 *in vitro.* Challenge of the snails with either type of phage prior to assaying the serum for the ability to neutralize phage does not induce an elevated level of this factor, and, in fact, snails previously challenged with T7 show decreased neutralization of T4D (Table I). Snails previously challenged with T4D have a higher titer than untampered snail serum when incubated with T4D *in vitro* but, due to the high standard deviation, this difference was not significantly different. These observations may indicate that the initial challenge of these snails with either T4D or T7 reduces the level of this factor in their hemolymph. It is noted that this naturally occurring factor appears at low levels in the serum since a 100-fold dilution of the serum prior to incubation with the bacteriophages reduces the neutralizing ability to the point that confluent plaques occur, as is observed when the phage are plated in the absence of serum.

Our finding of neutralization of T7 by *B. glabrata* serum is in contrast to that for the blue crab, where McCumber *et al.* (1979) observed that T3 and T7, two bacteriophages that are not cleared significantly *in vivo* (McCumber and Clem, 1977), are not neutralized *in vitro* whereas T2 phage is both cleared *in vivo* and neutralized *in vitro.* Furthermore, our finding of the ability of *B. glabrata* serum to neutralize T4D and T7 *in vitro* is in contrast to that for *C. virginica,* where Feng (1966) observed that serum from both noninjected oysters and oysters previously challenged with the bacteriophage *Staphylococcus aureus* phage 80 had no neutralizing effect on this bacteriophage *in vitro.* It is noted that Feng (1966) has reported that the clearance of *S. aureus* phage 80 is enhanced at higher ambient temperatures, *i.e.,* 15°C and 23.5°C as compared with 5°C.

The reduction in the mean serum titer of the bacteriophages T4D and T7 in the hemolymph of *B. glabrata,* therefore, appears to be due in part to a naturally occurring neutralization factor. However, since prior challenge of the snails with either bacteriophage does not stimulate higher levels of this factor, neutralization is probably not solely responsible for the more rapid reduction of phage titers *in vivo* in the serum of doubly injected snails. McCumber and Clem (1977), using radiolabeled viruses, have shown that T2, T4, and poliovirus are actively cleared from the circulation of the blue crab and not simply neutralized by the hemolymph. Although similar tracer studies have not been conducted in *B. glabrata,* the existence of an active, perhaps cell-mediated, clearance mechanism is the simplest explanation for our data. However, it is possible that some other neutralizing factor(s) are present in serum and are enhanced by previous challenge but are not demonstrable *in vitro, e.g.,* due to nonoptimal physiological conditions, the brevity of the assay, etc.

In conclusion, the data presented herein show that there is a reduction in the amount of infective bacteriophages in the hemolymph at 24, 48, and 72 h for T7 and at 24, 48, 72, and 96 h for T4D in doubly injected snails when compared to (1) snails that had been challenged with a single dose of coliphage and (2) snails that had been initially challenged with the Tris diluent or sham injected prior to challenge with bacteriophage. Thus, it would appear warranted to conclude that there is an enhanced ability in *B. glabrata* to clear and/or neutralize T4D and T7 *in vivo* that is inducible by previous homologous challenge. A comparison of *in vitro* and *in vivo* data implies that it is clearance rather than neutralization that is responsible for the enhancement effect. Whether this enhancement represents true "memory", *i.e.,* a separate, specific, and more rapid secondary response, or if it is simply a result of a prolonged primary response to the initial injection remains to be investigated.

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THE MOLECULAR WEIGHT OF RIBOSOMAL RIBONUCLEIC ACIDS AMONG THE PROTOSTOMIA

J. R. COLLIER

Biology Department, Brooklyn College, Brooklyn, NY 11210 and
Marine Biological Laboratory, Woods Hole, MA 02543

ABSTRACT

The molecular weights of the two larger rRNAs (18S and 26S) of one deuterostome and five protostomes were determined from their electrophoretic mobility. The molecular weight of the smaller (18S) ribosomal RNA was the same in all groups tested, but the molecular weight of the larger ribosomal RNA (26S) was 14 per cent smaller among the protostomes than the corresponding RNA (28S) of the deuterostomes.

INTRODUCTION

The size of ribosomal RNAs are moderately constant among the eucaryotes; however, the rRNA of the large subunit (the nominal 28S rRNA) is larger among mammals and birds and smaller among lower chordates and invertebrates (Perry *et al.*, 1970; Lewin, 1980). This pattern is well supported by analytical data for chordates and echinoderms, but there is a paucity of data among the Protostomia.

I report measurements of the molecular weights of the two larger rRNAs from five protostomes, a siliceous sponge (*Microciona prolifera*), an anemone (*Eloactis producta*), a nemertine worm (*Cerebratulus lacteus*), a mussel (*Mytilus edulis*), a clam (*Spisula solidissima*), and one deuterostome, the asteroid blood star (*Henricia sanguinolenta*). Observations showing the heat lability of the 28S rRNA of these invertebrates are also reported.

MATERIALS AND METHODS

RNA extraction

Animals were obtained from the Supply Department of the Marine Biological Laboratory, Woods Hole, MA. Either the entire specimen, or, for molluscs and asteroids, soft tissues, exclusive of the gut, were removed and homogenized at 4°C in a high-shear mixer with 5 ml of homogenizing solution (1% triisopropylnapthylenedisulfonate, 1% sodium dodecyl sulfate, 0.1% Macaloid, 10^{-2} *M* magnesium acetate, 10 μg/ml polyvinylsulfonate in 0.01 *M* sodium acetate, pH 5.0) and 5 ml of phenol per g of tissue. The homogenate was centrifuged, the aqueous phase recovered, and extracted again with phenol. The final aqueous phase was recovered by centrifugation and precipitated by the addition of NaCl to 0.1 *M* and 2 volumes of 95% ethanol and storage at −20°C overnight. RNA for molecular weight standards was extracted from rat liver and *E. coli* by the same procedure.

The heat lability of rRNA was determined by heating a sample of RNA from each specimen for 5 min at 60°C. After electrophoresis, as described below, the area under the gel scan was measured and the per cent of the 28S rRNA that degraded was calculated by comparison with an unheated preparation of rRNA.

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Electrophoresis

The precipitated RNA was dissolved in 0.04 *M* tris-acetate buffer, pH 7.4, and layered over a 2.4 per cent acrylamide gel contained in a 6 × 0.5 cm glass tube

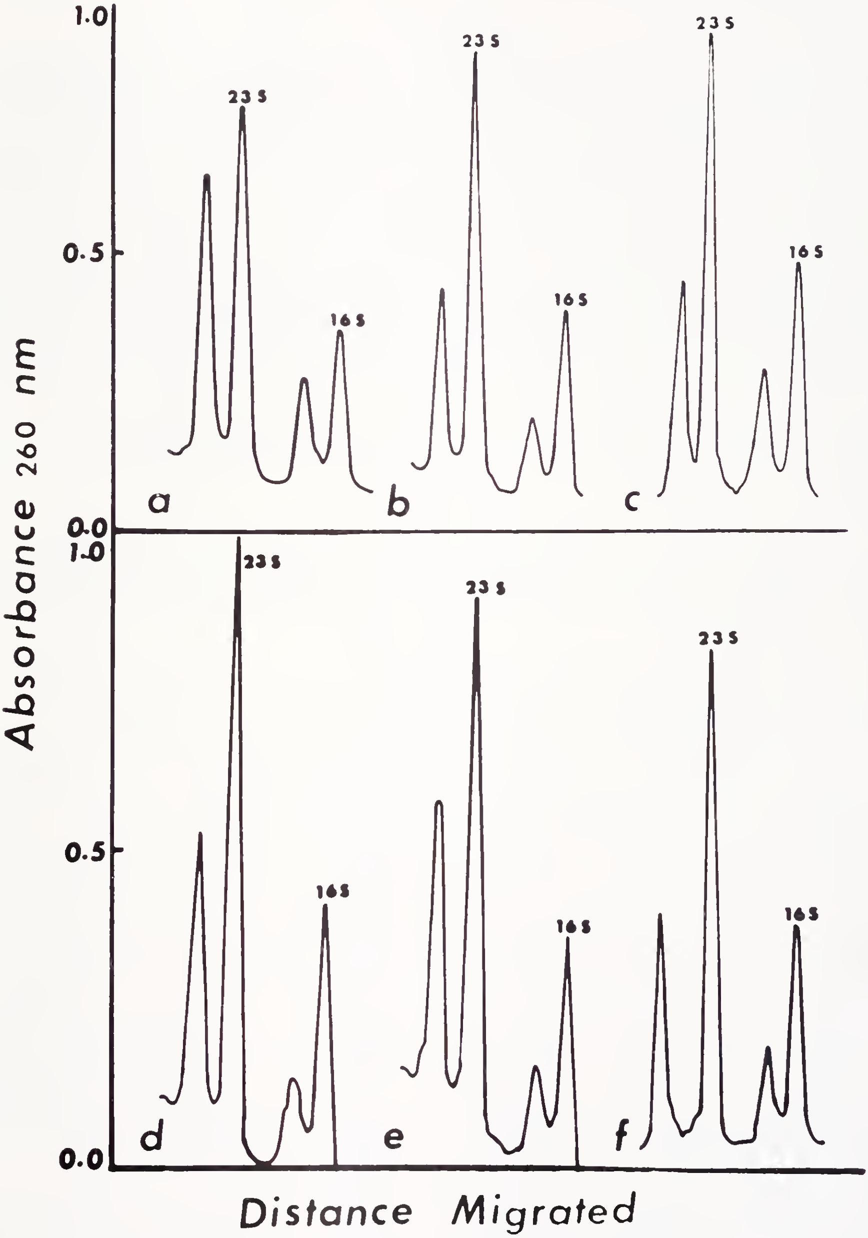

FIGURE 1. Relative mobility of RNA samples and RNA standards. The peaks marked 16S and 23S are RNA standards from *E. coli*. Ribosomal RNAs are from: (a) *Microciona prolifera*, (b) *Eloactis producta*, (c) *Cerebratulus lacteus*, (d) *Spisula soldissima*, (e) *Mytilus edulis*, and (f) *Henricia sanguinolenta*.

(Loening, 1968). Electrophoresis was at a constant current for 5 mA per gel for 45–60 min in a buffer of 0.04 *M* tris-acetate, pH 7.4, containing 0.2% sodium dodecyl sulfate. After electrophoresis the gels were placed in a quartz cuvette and scanned at 260 nm in a Gilford spectrophotometer.

RESULTS

RNA standards were co-run with each experimental sample and the relative mobility of each sample and its set of standards was plotted against the log molecular weight of the standards. Relative mobilities were determined by measuring the distance each species of RNA migrated into the gel (Fig. 1). The molecular weights of the unknown RNAs were calculated from the linear relation between molecular weight and relative mobility of the RNA standards. That these RNAs were ribosomal RNA was evident from their size and abundance.

Table I contains replicate measures, average molecular weights, and standard errors for the two larger rRNAs of each of the six species studied. Among these were one deuterostome, a starfish, and five protostomes (two molluscs, a nemertine worm, an anemone, and a sponge). Also in Table I, column 4, are the per cent degradation of the 28S rRNA caused by heating at 60°C for 5 min. The 18S rRNAs of all species were stable when heated at 60°C for 5 min. It is not clear why the 26S rRNA of *Mytilus, Spisula,* and *Cerebratulus* were completely degraded by heating at 60°C (Table I).

DISCUSSION

The molecular weight of 18S rRNA does not vary significantly among the species listed in Table I. The size of this RNA has also been found to be constant among

TABLE I

Molecular weight and thermal stability of ribosomal RNAs

	Molecular weight × 10^{-6}				
Organism	Large rRNA		Small rRNA		% degradation of 26 S RNA
Henricia	1.56		0.70		16
sanguinolenta	1.59	1.59	0.71	0.70	
	1.61	± 0.015*	0.69	± 0.006	
Spisula	1.37		0.70		100
soldissima	1.38	1.38	0.70	0.70	
	1.40	± 0.009	0.71	± 0.003	
Mytilus edulis	1.40		0.69		100
	1.40	1.39	0.69	0.68	
	1.38	± 0.007	0.67	± 0.007	
Cerebratulus	1.32		0.71		100
lacteus	1.33	1.33	0.71	0.72	
	1.33	± 0.003	0.72	± 0.003	
Eloactis	1.38		0.70		21
producta	1.37	1.38	0.70	0.70	
	1.38	± 0.003	0.71	± 0.003	
Microciona	1.36		0.71		11
prolifera	1.37	1.37	0.71	0.71	
	1.37	± 0.003	0.72	± 0.003	

* Mean and standard error of mean.

other organisms in which it has been measured (Table II). In contrast to this consistency in the size of 18S rRNA throughout the animal kingdom, the molecular weight of the larger rRNA of the protostomes is 14 per cent smaller than the corresponding rRNA of the single deuterostome reported in Table I and of those deuterostomes previously studied (Loening, 1968; Attardi and Amaldi, 1970; Perry *et al.*, 1970; and Lewin, 1980). However, the size of the larger rRNA is invariant among the protostomes observed and among representatives of two groups, sponges and coelenterates, of their immediate phylogenetic predecessors. Therefore, it appears that a major increase in the size of the RNAs of the large ribosomal subunit occurred as the deuterostomes diverged from the protostomes, and that other changes in size did not arise among the older protostomes or their immediate predecessors.

Among the protozoa, with the exception of *Acanthamoeba* (Loening, 1968), the larger RNA is 5.8% smaller than the average size of this RNA among the protostomes. (The size of the *Acanthamoeba* rRNA is uncertain as the RNA used for molecular weight determination was unstable (Loening, 1968).) While the protozoan rRNA may be smaller than the protostomian rRNA there is not enough data avail-

TABLE II

Molecular weights of invertebrate ribosomal RNAs

Organism	Molecular weight × 10^{-6}		Reference
	Large rRNA	Small rRNA	
Protozoa			
Acanthamoeba	1.53	0.89	Loening (1968)
Euglena gracilis	1.30	0.85	Rawson and Stutz (1968)
Tetrahymena pyriformis	1.30	0.69	Loening (1968) Kumar (1969)
Paramecium	1.31	0.69	Loening (1968) Reisner *et al.* (1968)
Porifera			
Microciona prolifera	1.37	0.71	Table I
Coelenterata			
Eloactis producta	1.38	0.70	Table I
Nemertina			
Cerebratulus	1.33	0.72	Table I
Nematoda			
Ascaris lumbricodes	1.42	0.76	Tobler *et al.* (1974)
Arthropoda			
Drosophila	1.40	0.73	Loening (1968)
	1.40	0.68	Hastings and Kirby (1966)
	1.40	0.65	Perry *et al.* (1970)
Mollusca			
Spisula solidissima	1.38	0.70	Table I
Mytilus edulis	1.39	0.68	Table I
Mulinia lateralis	1.34	0.75	Kidder (1976)
Ilyanassa obsoleta	1.37	0.70	Koser and Collier (1971)

able to establish that this difference is significant. Thus, the possibility remains that an increase in the size of the RNA of the large ribosomal subunit occurred before or at the time of the appearance of the sponges.

From estimates of sequence diversity of rRNA from a number of organisms Pinder *et al.* (1969) found that the structure, but not the exact sequence, of rRNA was conserved during evolution. These observations suggest that changes in nucleotide sequence of rRNA are tolerated as long as the overall size and shape of the rRNA is suitable for the assembly of a ribosome that will function efficiently in protein synthesis. Thus, the reduced size of the protostomian rRNA is suitable, as is the still smaller rRNA of procaryotes, for the construction of a satisfactory ribosome. It may be found, however, that the ribosomal proteins of protostomian ribosomes are different, as are the proteins of procaryotic ribosomes, from deuterostomian ribosomes.

The degradation of the 26S rRNA after heating at 60°C (Table I), as has been previously reported for a number of organisms (Koser and Collier, 1971; Shine and Dalgarno, 1973), indicates that one or more fragments of RNA are hydrogen-bonded to the main 26S rRNA component. The data reported here show, for the first time, that these rRNA fragments exist in a sponge and an anemone, which suggests that this organization of rRNA existed before the appearance of the protostomes.

ACKNOWLEDGMENTS

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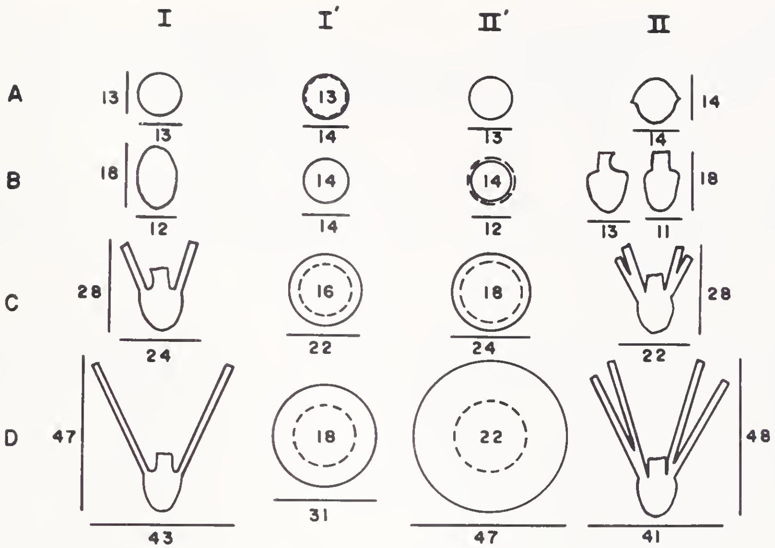

FIGURE 2. Drag and surface area indexes for larval models. Columns I and II contain eight different models for which drag was measured empirically. Columns I' and II' show Stokes' diameters of spheres, plotted as solid circles, which have equivalent drag to models in the adjacent columns. The dotted circles represent spheres which have equivalent surface area to models in the adjacent columns. Numbers on the models refer to the models' dimensions, in mm (50× life size); numbers inside the circles refer to the diameter of equivalent surface area spheres. Numbers below the circles refer to diameters of spheres of equivalent drag.

L = length of arm = 320, 800, or 1600 μm

V = swimming velocity = 10^{-3} m/s

a = radius of arm = 10 μm

A swimming velocity of 1 mm/s is probably high for a larva with 1600 μm long arms, but the plutei of species mentioned above swim at or near this speed (Emlet, pers. ob.). Since no information exists on swimming velocity changes with size in plutei, I assumed 1 mm/s for all sizes. Drag on the arms must be summed with body drag (scaled using Stokes' diameter for the empirical measurements discussed above). Figure 3 shows the relationship between equivalent Stokes' diameter, arm length, arm number, and arm orientation.

The model for forces generated in swimming

To test the hypothesis that fenestrated spicules are necessary to support longer arms, forces produced during swimming were calculated and resolved into bending forces on the arms. According to the ciliary sublayer model (Blake, 1972; Roberts, 1981), the total force required to generate the ciliary currents during swimming has two components, F_c and F_d. F_c (ciliary force) is the force required to maintain the shear gradient in the ciliary layer. Since the fluid at the tips of the cilia shears with respect to fluid external to it, there is the additional component of drag, F_d (drag force), whose maximum value is the drag on the inert organism (with cilia) being pulled at swimming velocity through the medium (Roberts, 1981; Sleigh and Blake, 1977). The determination of this latter component has already been discussed above.

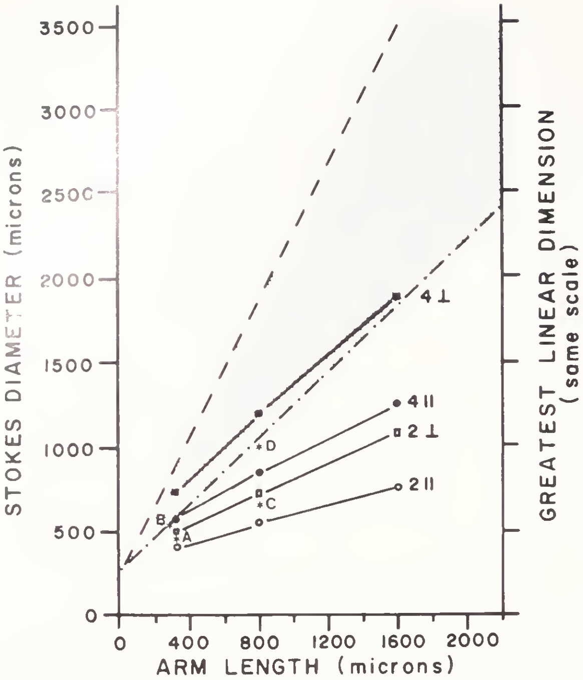

FIGURE 3. Expressed as Stokes' diameter, drag increases with arm length in two armed and four armed larvae. Reynolds numbers range from 0.35 to 1.3. 2 ‖, two arms parallel to flow; 4 ‖, four arms parallel to flow; 2 ⊥, two arms normal to flow; 4 ⊥, four arms normal to flow. The transformed empirical drag measurements (Fig. 2) are shown as (*). A*, two 320 μm long arms; B*, four 320 μm long arms; C*, two 800 μm arms; D*, four 800 μm long arms. The shaded area of the graph is the range of greatest linear dimensions for larvae which move arms from a parallel (–·–·–) to a perpendicular (– – –) orientation. The y-intercept is the diameter of the body region, approximately 260 μm.

I have modified this model for swimming in plutei and made the following assumptions. I assume that the ciliated band along the arms produces all of the locomotory forces and that the velocity profile is linear between the organism's surface and the cilia tips (Fig. 4). Reverse flow in the recovery stroke region is ignored, but Roberts (1981) shows that there is no reverse flow in this region in a freely swimming organism. Cilia in the band are approximately 25 μm long, and the band is 10 μm wide. These assumptions, the use of passive drag as a maximum estimate of the F_d component, and other assumptions to be mentioned later maximize the forces and so maximize the predicted bending of the arms. Thus the hypothesis that stiffer spicules are necessary to prevent bending in longer arms is favored. Under this model rejection of the hypothesis is a robust result, acceptance of the hypothesis is an inconclusive result.

F_c, the force required to maintain the velocity gradient between the organism's surface and the tips of the cilia, is found by solving the equation which defines viscosity.

$$F_c = \mu S U_x / l$$

where

μ = dynamic viscosity of the fluid

S = surface area over which the gradient is maintained

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LOCOMOTION, DRAG, AND THE RIGID SKELETON OF LARVAL ECHINODERMS

RICHARD B. EMLET

Department of Zoology, University of Washington, Seattle, Washington 98195, and Friday Harbor Laboratories, University of Washington, Friday Harbor, Washington 98250

ABSTRACT

A model for the forces generated by a swimming pluteus larva is constructed by characterizing flow over the ciliated band and experimentally determining drag on different larval shapes. These and other mechanical calculations are used to examine a hypothesis on mechanical variation of the larval skeleton. Drag on a larva increases with arm number and arm length and with increasingly perpendicular orientation of arms to flow. Drag on arms may reduce sinking rate. Of the two types of skeletal rods found in plutei, fenestrated arm spicules have a flexural stiffness three to five times greater than simple arm spicules. Fenestrated spicules are associated with longer arms in plutei, but they may not be necessary to prevent deflection of arms under forces produced by ciliary locomotion. Other alternatives which may explain fenestration are discussed.

INTRODUCTION

> Since the larval lophophore of ACTINOTROCHA is supplied
> With a pair of 'collar' coelomes, these can scarcely be denied
> To former Echinozoic fry, which hydraulic pressure needed
> To support their lateral armlets ere a skeleton succeeded.
>
> Garstang, 1966, p. 52.

The pluteus larvae of echinoids and ophiuroids are large (0.5–2.0 mm) and elaborately shaped mobile plankters characterized by anteriorly directed arms and an internal rigid skeleton. The arms are cylindrical extensions of the epidermis and each contains a skeletal spicule shaped as a cylindrical or fenestrated beam and composed of magnesium calcite (Okazaki and Inoué, 1976). These animals capture food and swim by means of a band of cilia which courses the length of the arms and the circumference of the main body region. Throughout larval life, prior to metamorphic competence, the number and lengths of the arms and spicules increase, and the length of the ciliated band increases. The mechanical consequences of growth for a larva are increased total drag forces and increased forces on the arms. This paper applies scaling principles of fluid mechanics to irregular larval forms and presents a model for swimming/feeding forces generated by larvae. This model is a modification of the ciliary sublayer model (Blake, 1972; Blake and Sleigh, 1974; Roberts, 1981) and allows calculation of forces generated by the ciliated band. One of the components of the model, passive drag force, is measured empirically and is compared for different larval shapes. The swimming model is used to test the hypothesis that forces generated in current production require stiffer spicules in longer armed larvae accounting for skeletal variation amongst plutei.

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Ciliary swimming

With few exceptions ciliary locomotion occurs at Reynolds numbers below unity, where viscous forces control fluid behavior (Sleigh and Blake, 1977; Vogel, 1981). Ciliated organisms swim through a liquid by moving fluid past the ciliated surface at speeds about twice swimming velocity (Blake and Sleigh, 1974; Roberts, 1981). Flow profiles around a swimming ciliated organism are different than those around a similar organism being pulled through a fluid. For an object being pulled (or pushed) through a stationary fluid, flow velocity decreases monotonically from free-stream velocity to zero velocity at the object's surface. For an actively swimming organism, velocity increases from free-stream velocity to some maximum at or near the tips of the cilia and then decreases approximately linearly until it reaches zero at the outer edge of the recovery stroke region, the region where cilia fold close to the organism's surface, and return to position for the power stroke (Cheung and Winet, 1974).

In a pluteus the ciliated band produces the currents for both swimming and feeding. Strathmann (1971, 1975) described the feeding mechanism and showed the directions of currents over the arms and ciliated band. Water from a medial anterior position moves radially and posteriorly across the ciliated band. The arms must withstand the reaction forces to those which the cilia exert on the water. Under these forces the arms would tend to collapse medially. Any decrease in the distance in the arm to arm width would decrease the posterior component of water movement and may also reduce the area of the water sampled by the feeding organism. The consequences would be reduced swimming and feeding efficiencies. As echinoid larvae grow older, they often develop epaulettes or vibratile lobes. These specialized regions of the ciliated band have longer cilia than the rest of the ciliated band and are located on the body at the base of the arms. In swimming, they push the body and arms through the water and therefore indirectly create an outward force on the arms which opposes the force of the ciliated band.

Larval skeleton

Around the time of gastrulation two triradiate spicules form and grow into highly characteristic skeletal elements which are continually modified by secretion or absorption of calcitic material (Fig. 1A, B). In its final stage an echinopluteus (echinoid pluteus) contains up to eight separate skeletal rods. Depending on species and location in the body, these spicules may be simple cylindrical rods or fenestrated rods (Fig. 1A–E). Emlet (1982) measured the bending stiffness of these two types of spicules and found the fenestrated ones to be three to five times stiffer than the simple ones. It is unknown why homologous skeletal spicules are fenestrated in some species and simple in others. One possibility is that there is a need for stiffer spicules in longer arms to prevent bending from the forces generated by ciliary locomotion. I discuss this possibility with measurements from the literature and calculations from the swimming model presented below.

In echinoplutei of clypeasteroids and older families of regular echinoids the skeleton plays a role in movement of certain pairs of arms (postoral and posterodorsal, Fig. 1A, B). These arms are also the ones which contain spicules that may be either simple or fenestrated. Muscles attach to the base of these spicules and allow a larva to move one to four arms between positions parallel and perpendicular to the longitudinal body axis. This movement causes shape changes which influence drag and will be analyzed below. Some movement also occurs in other pairs of arms (the preoral and anterolateral arms, see Fig. 1A, B) in association with contractions

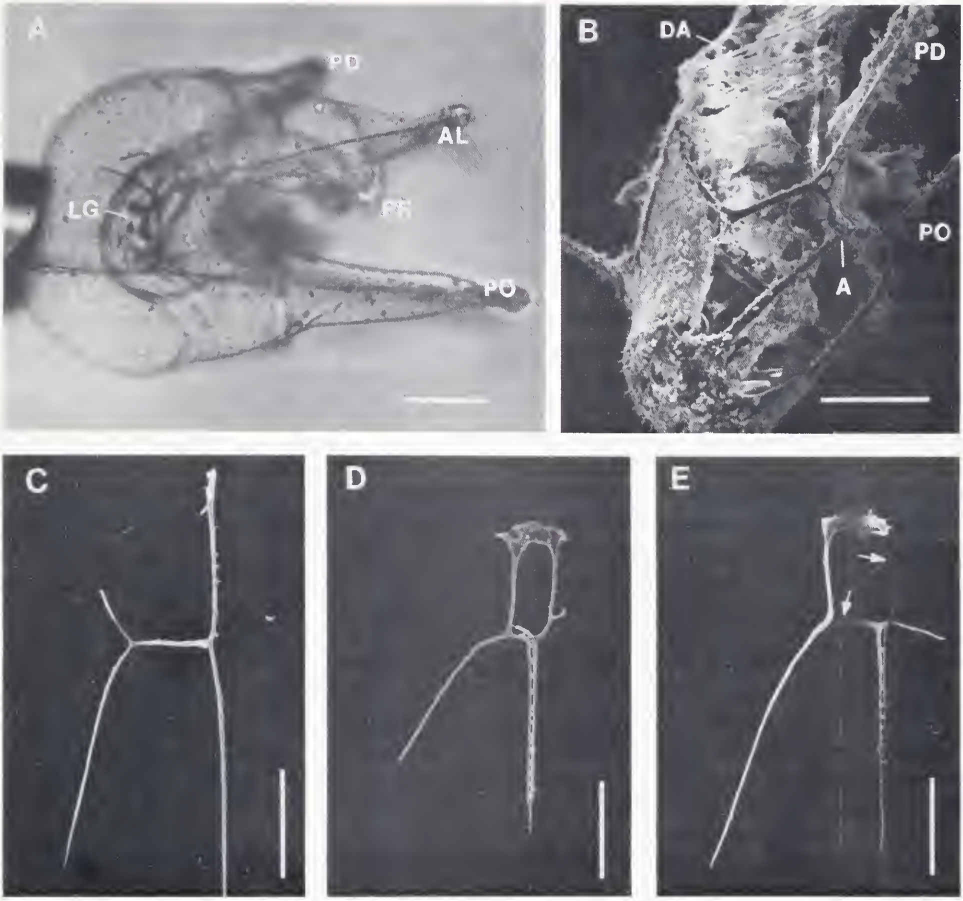

FIGURE 1. Echinoplutei and their spicules. (A) Lateral view of a two week old larva of *S. franciscanus* held in place by a suction pipette. Note simple spicules. (Transmitted light micrograph) Scale: 100 μm. (B) Dorsolateral view of a 3 week old larva of *D. excentricus* with fenestrated spicules showing through a torn epidermis. Scale: 50 μm. (C) 'Half-skeleton' of *S. franciscanus* showing the simple arm spicules, same stage as A. (D, E) Early and later stages of the fenestrated 'half-skeleton' of *D. excentricus*. E. Arrows show where the skeleton is modified to allow articulation of the fenestrated postoral rod. C, D, E scale: 100 μm. (B–E are SEM micrographs.)

Key: AL = anterolateral arm and spicule, DA = dorsal arch spicule, PD = posterodorsal arm and spicule, PO = postoral arm and spicule, PR = preoral hood region, LG = larval gut, A = articulation of spicules.

of the preoral dilator musculature. This latter type of arm movement is associated with handling and rejecting food particles as discussed by Strathmann (1971).

In contrast to the echinoplutei, ophioplutei have only two spicules and these are not movable. Each of these spicules branches into four to six simple rods in fully developed larvae.

MATERIALS AND METHODS

The larvae used in this study were raised in culture or obtained from the plankton and include the species *Clypeaster subdepressus* (Gray), *Dendraster excentricus* (Eschscholtz), *Diadema mexicanum* A. Agassiz, *Echinometra vanbrunti* A. Agassiz, *Strongylocentrotus droebachiensis* (O. F. Muller), *S. franciscanus* (A. Agassiz), *S. purpuratus* (Stimpson) and *Toxopneustes roseus* (A. Agassiz). Larvae were observed carefully, and dimensions of larvae and spicules were noted for calculations which follow.

Passive drag determination

The passive drag forces, F_d, on irregularly shaped larval forms were determined experimentally as follows. Models, 50 times life size, of a sphere, a prolate spheroid, and a pluteus body with interchangable arms of two lengths (equivalent to 320 and 800 μm) were made from brass and steel. Drag forces on these models were determined at Reynolds numbers between 0.3 and 0.7, the same as those experienced by larvae. Comparable Reynolds numbers mean that flow conditions around the models are dynamically similar to those around larvae pulled through a medium (Vogel, 1981). A special high viscosity flow tank (designed by S. Vogel) containing partially diluted corn syrup (dynamic viscosity 1.2–1.9 kg/m s) was used. Models were held by a sting which was attached to a force transducer, and drag force for each model was measured at three flow velocities.

At Reynolds numbers less than unity, Stokes' law can be used to convert drag measurement into Stokes' radius or, as I have presented here, Stokes' diameter. This value is the radius or diameter of a sphere which has the same drag as the irregular shape for which it is determined and provides an index for comparing different irregular shapes.

$$F_d = 6\pi\mu rV = 3\pi\mu dV$$

where

μ = dynamic viscosity

r = Stokes' radius

V = swimming velocity

d = Stokes' diameter

Because the flow conditions around the models and the larvae are dynamically similar the passive drag force on a larva may be determined. The dimensionless ratio of the model's Stokes' diameter to the model's length is constant over all sizes. Since the lengths of the model and the larva and the model's Stokes' diameter are known, the Stokes' diameter for a larva can be calculated. Substituting this value and the values of viscosity of sea water and the swimming velocity of a larva into the equation above will give a value for F_d of a larva.

Drag forces of the different larval shapes are compared through their empirically determined Stokes' diameters. Figure 2 shows the Stokes' diameters, represented as circles, drawn next to the shape which has equivalent drag. Surface areas are also calculated to compare with shapes and drags.

Since I found drag forces to be additive (see Results), I determined the effect of arm number, arm length, and arm posture on passive drag for an appropriately scaled larval shape. Arms were treated as cylinders parallel or perpendicular to flow, and drag force on the arms of three lengths (320, 800, and 1600 μm) was calculated with the following formulae (Vogel, 1981).

$$F_{\parallel} = 2\pi\mu LV/[\ln(L/a) - 0.807]$$

$$F_{\perp} = 4\pi\mu LV/[\ln(L/a) + 0.193]$$

where

μ = dynamic viscosity of sea water: 1.39×10^{-3} kg/m s

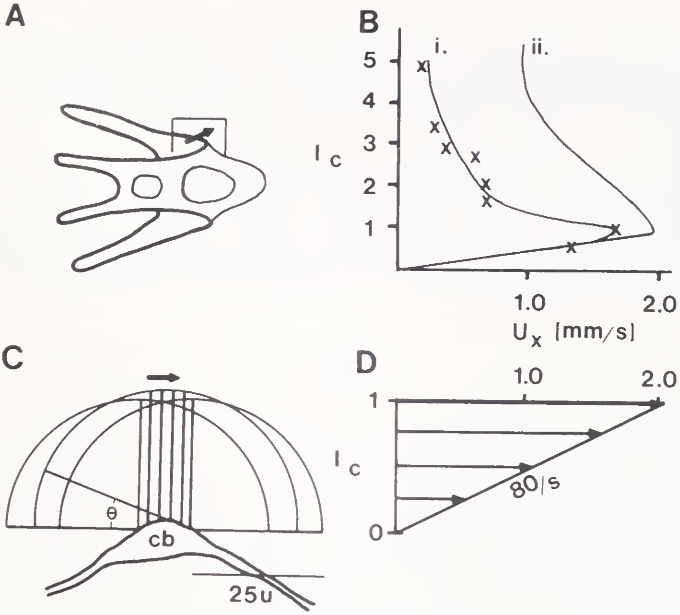

FIGURE 4. Description of flow across the ciliated band. (A) Dorsal view of a four armed pluteus. The heavy line circumscribing the arms indicates the location of the ciliated band. The box indicates the region on which B, C, and D focus. The arrow shows the direction of water movement. (B) Velocity profile to five cilium lengths above the larval body. l_c: unit length of cilium. U_x: Fluid velocity parallel to organism's surface. Curve (*i.*) Flow profile from films of a tethered pluteus by Strathmann *et al.* (1972). Curve (*ii.*) Idealized flow for a free swimming organism (*e.g.* Roberts, 1981). (C) Cross-section through the ciliated band (cb). Cilia are free to swing in 180° arcs. θ is the angle a cilium makes with the organism's surface. In this region the ciliary shear gradient is established. (D) The shear gradient ($U_x/l = 80/s$) in the ciliated region assumed for the swimming model. U_x and l_c are the same as in B. l is the distance in μm above the organism's surface (max is the cilium length, l_c, 25 μm).

U_x = velocity of the fluid in direction parallel to the band surface

l = distance above the arm surface (max. is the cilia length, l_c, 25 μm).

U_x/l is the velocity gradient perpendicular to the organism's surface and is maintained across the band in the 180° arc of the cilia movement. Assume $U_x = \omega l_c \sin \theta$, where ω is angular velocity, l_c is cilium length, and θ is the angle between a cilium and the organism's surface, such that $l = l_c \sin \theta$. Then the gradient is conveniently ω. Measurements from high speed films of plutei (taken by Strathmann *et al.*, 1972) show a fluid velocity at the tips of the cilia of 1.7 mm/s at room temperature, but 2.0 mm/s is used to weight the test in favor of larger forces (Fig. 4B). Thus, under these assumptions, the angular velocity and the velocity gradient is 80/s and is maintained over the surface 60 μm wide (25 + 10 + 25 μm) along two sides of an arm of known length (Fig. 4C, D).

I restrict this model to the cases of ophioplutei with two arms and echinoplutei with four arms because these are the conditions for which I measured the passive drag component. There is no reason that the calculations cannot be done to take into account more arms. In the case of a four armed pluteus, each arm produces 1/4 of the F_d component and its own F_c component. Thus the maximum net force, F_n, on any one arm is:

$$F_n = F_c + F_d/4$$

The maximum tip deflection of an arm of length, L, assuming the force is evenly distributed along its length is (Gordon, 1978):

$$Y_{max} = F_n L^3/8(E_s I_s)$$

where E_s and I_s are the Young's modulus and second moment of area determined for the spicules (see Materials and Methods in Emlet, 1982). I assume a zero stiffness of the epithelium on the arm. Though the epithelium surely has stiffness, this assumption only increases the forces on the spicule and makes the test more robust.

RESULTS

Studies on drag

The studies performed to calculate F_d show the effect of shape on drag. The sphere, prolate spheroid, and pluteus body without arms all have similar drags (Fig. 2, row A and B), but drag on the pluteus increases with arm number and length (Fig. 3). Drag approximately doubles with a doubling of arm length in four arm models (Fig. 2, column II', row C and D). Drag does not increase this rapidly for two arm models (Fig. 2, column I', row C and D). If the spheres of equivalent surface area are calculated and compared with drag for each of the models, drag increases more rapidly than surface area (Fig. 2, column I' and II', solid versus dotted circles). These data show that form is important in determining drag at Reynolds numbers near unity.

Drag on a larva was found to be an additive property of its parts. The expected drag was calculated for the long four armed model by treating the arms as four cylinders parallel to flow and adding the drag on the body only. The calculated drag gives a Stokes' sphere of 47.7 mm diameter, while the empirically determined Stokes' diameter is 47.2 mm. The empirically determined drags of all the pluteus models, transformed into larval proportions are shown in Figure 3 (∗) and agree well with expected drags. The oblique orientation of the arms on the empirical models explains their intermediate values of drag between those expected for larvae with arms oriented parallel or perpendicular to flow.

Figure 3 also shows the change in drag due to change in arm orientation and length. These results show that spreading arms increase drag proportionately more in larvae with longer arms, especially in four armed larvae. The range of Reynolds numbers for these calculations is 0.35 to 1.3, when overall length is the dimension used to calculate Reynolds number. For arm lengths greater than 1100 μm ($Re > 1.0$), the lines are approximate; the formulas used for calculation of drag are no longer strictly applicable.

Forces on arms and predicted deflections

The forces required to generate and maintain the shear gradient and those necessary to overcome passive drag were taken as an upper limit to the force acting on the spicules. Table I shows the forces due to the cilia and body shape for two armed and four armed larvae and deflections expected in spicules of three different lengths and two stiffnesses. The deflections are all small. In a four armed larva deflection at the tips of arms 800 μm long is 2.4 μm for simple spicules and 0.6 μm for fenestrated spicules. If the lengths were doubled again and the increased forces calculated as before, the expected deflection of a 1600 μm simple spicule is 36 μm

TABLE 1

Forces and calculated deflections for larval arms in four armed and two armed larvae with three different arm lengths and two spicule types

Arm length (μm)	F_c	F_d	Deflection simple spicules	Deflection fenestrated spicules
		Four armed larvae		
320	4.3×10^{-9} N	6.8×10^{-9} N	0.06 μm	0.02 μm
800	10.7×10^{-9} N	13.5×10^{-9} N	2.4 μm	0.6 μm
1600	21.4×10^{-9} N	20.2×10^{-9} N	35.6 μm	9.6 μm
		Two armed larva		
320	4.3×10^{-9} N	6.2×10^{-9} N	0.08 μm	0.02 μm
800	10.7×10^{-9} N	8.9×10^{-9} N	2.6 μm	0.7 μm
1600	21.4×10^{-9} N	11.9×10^{-9} N	24.1 μm	6.5 μm

and of a fenestrated spicule is 10 μm. For 1600 μm long arms set at 30° to the longitudinal axis, a 36 μm deflection is less than 5% of the distance between the arms, and the area between the deflected arm tips is 91% of the original area. For the 320 and 800 μm long arms, deflections are similar in the two armed and four armed larvae. This similarity occurs, even though passive drag on the two armed form is less, because only two arms share the total swimming force. As arm length increases beyond 800 μm, passive drag does not increase as rapidly for the two armed larva as for the four armed larva. Thus for 1600 μm long arms, deflections are less in a two armed larva than in a four armed larva. It appears that for most species the added stiffness provided by fenestration is not necessary to prevent deflection created by the usual ciliary forces.

Epaulettes or vibratile lobes will indirectly counteract forces due to the cilia on the arms. If arms are treated as cylinders with axis of rotation normal to flow and drag is calculated for a current velocity of 1 mm/s, the deflections may be calculated. This will approximate the condition in which epaulettes or vibratile lobes propel a larva with maximum drag while the arms are producing no current. For arms with a simple rod the deflections in the posterior direction are 0.02, 0.5, and 6.8 μm for the 320, 800, and 1600 μm long arms respectively. These calculations show that epaulettes can substantially reduce bending in arms due to currents generated by the ciliated band.

DISCUSSION

The results from the passive drag studies show that drag increases with arm number, arm length, and increasingly perpendicular orientation of arms to flow (Fig. 3). Mortensen (1921) suggested arm movement in plutei is important to slow sinking rates. My results show that arms and arm flaring behaviors should slow sinking rate by increasing drag. This effect should be most enhanced in organisms with longer and more numerous arms, but whether larvae actually flare arms to slow sinking rate is unknown.

The ends of an object, regardless of orientation to flow, usually experience different flow conditions and patterns than other parts of the object. These differences, called end effects, often result in increased drag on the object. In this study, the similarity between empirical (flow tank) and expected (calculated) values for drag shows that end effects on the individual parts are negligible and that drag may be determined additively by summing drags of regularly shaped components. These results have two implications. First, for flow conditions similar to those used in this study, drag on any irregular shape may be calculated if these shapes can be broken into regular shapes for which drag formulae are known. Second, evaluation of drag will allow density changes to be estimated in living larvae even if size and shape change with age. The sinking rate of an anesthetized larva is a function of its passive drag and density. Since drag can be estimated, density may be calculated by the Stokes' formula for terminal velocity (see *e.g.* Vogel, 1981).

Skeletal variation in echinoplutei

Spicules may support the arms and prevent deflections from forces created by production of swimming and feeding currents. However, comparison among larvae and lophophorates shows that ciliated extensions subject to forces similar to those experienced by larval arms do not have spicules. Examples include the bipinnaria larva of asteroids, the actinotroch larva of phoronids, and the lophophore tentacles of adult phoronids and bryozoans.

If spicules do support the arms against deflections from currents, then longer arms, which are subject to greater forces, should have stiffer skeletons. Table II

TABLE II

Species, arm lengths (postoral arms), and spicule type of larvae which have been drawn to scale

Species	Arm length (μm)	Spicule type	Reference
Arbacia sp.	5000	F	Mortensen, 1921.
Eucidaris metularia	1850	F	Mortensen, 1937.
Prionocidaris baculosa	1750	F	Mortensen, 1938.
Echinocardium cordatum	1071	F	Mortensen, 1931.
Diadema setosum	0953	F	Mortensen, 1931.
Arbacia stellata	0950	F	Mortensen, 1921.
Echinus esculentus	0711	S	MacBride, 1903.
Heterocentrotus mammillatus	0708	F	Mortensen, 1937.
Echinometra mathaei	0617	F	Mortensen, 1937.
Echinometra luncunter	0600	F	Mortensen, 1921.
Mespilia globulus	0600	F	Mortensen, 1921.
Laganum depressum	0600	F	Mortensen, 1931.
Evechinus chloroticus	0590	F	Mortensen, 1921.
Echinus miliaris	0589	S	MacBride, 1903.
Clypeaster humilis	0572	F	Mortensen, 1937.
Lytechinus varigatus	0567	S	Mortensen, 1921.
Echinodiscus auritus	0565	F	Mortensen, 1938.
Temnotrema scillae	0539	F	Mortensen, 1937.
Temnopleurus sp.	0533	F	Mortensen, 1921.
Strongylocentrotus franc.	0510	S	Emlet, lab data.
Heliocidaris tuberculata	0500	F	Mortensen, 1921.
Strongylo. pulcherrimus	0500	S	Onoda, 1936.
Echinocyamus pusillus	0500	F	Mortensen, 1921.
Astriclypeus manni	0467	F	Mortensen, 1921.
Tripneustes esculentus	0433	F-S	Mortensen, 1921.
Strongylo. purpuratus	0367	S	Strathmann, 1971.
Dendraster excentricus	0304	F	Emlet, lab data.
Strongylo. droebachiensis	0217	S	Emlet, lab data.

Scaled drawings of later stage larvae were chosen to avoid shorter lengths of younger larvae. For spicule type F = fenestrated, S = simple, F-S = both fenestrated and simple.

contains a list of later stage echinoplutei (with six and eight arms) which were drawn to scale by Mortensen and other authors. The species are listed in order of decreasing arm length (Column 2). Comparisons among echinoid larvae show that the stiffer fenestrated spicules do occur in larvae with longer absolute arm length (Mann-Whitney U test, $P < 0.01$).

To examine the hypothesis of support in swimming more rigorously, I modeled the expected forces on the arms during swimming. The results (Table I) show that deflections in arms which contain simple spicules are very small. This model contains several assumptions (see Methods) which increase the forces acting on the spicules and so may overestimate expected bends. This approach increases the robustness of the conclusion that fenestrated spicules may not be necessary to prevent bending even in arms as long as 1600 μm. Epaulettes and vibratile lobes should also reduce the likelihood of bending due to forces created by the ciliary band on the arms. I have never observed bending in any spicules which could be attributed to ordinary ciliary forces.

Under a given force, arm deflection at the tip is directly proportional to the arm length cubed so deflection in the longest arms may become important. Mortensen (1937) mentions larvae of a West Indian diadematid, *Echinopluteus transversus*, with arms as long as 12 mm. Diadematid and arbaciid larvae have arms long enough that bending might become important. Ophioplutei often have a pair of arms two to three mm long which should experience forces great enough to cause bending. Though the arms of these larvae contain simple spicules, they are more massive at the bases than those in echinoplutei and taper to small diameter at the tips. The different shape of ophiuroid spicules may be another way to stiffen long arms.

Another suggested function of the skeleton might explain the variation in spicule type. Gustafson and Wolpert (1961) propose that the function of the spicules is to extend the larval arms. If the epidermis can produce enough tension to approach the bucking force of the rods, stiffer fenestrated rods could help prevent buckling. Since the force needed to cause buckling is inversely proportional to the square of rod length, longer spicules are more likely to buckle, and this would explain the correlation between fenestrated spicules and long arms. There is probably some tension in the epidermis because it retracts down the spicule at the arm tips when they are damaged. To examine this possibility further tension in the epidermis would have to be measured. Incidently, this tension may also stiffen the arms as guy wires stiffen a pole.

It is possible that the arms and spicules function as defensive structures in a manner analogous to that of chaetae of some polychaete larvae (Wilson, 1929). Spicules stiffen the arms and body, and this may prevent ingestion by some predators. Arm movements may also help prevent a larva from being eaten by increasing its diameter. By spreading arms to a perpendicular position a larva increases its greatest linear dimension two-fold. The shaded region of Figure 3 shows how the linear dimension changes with arm orientation. Longer arms increase the absolute magnitude of dimension change.

Arm flaring in larvae appears to be associated with larvae that have fenestrated spicules. I have observed larvae of *Dendraster* and of an unidentified spatangoid spread their arms when touched or pulled by suction. Both these species have fenestrated rods in their movable arms. Other species which fit this pattern are *Clypeaster subdepressus, Diadema mexicanum, Echinometra vanbrunti.* Mortensen (1921, 1937, 1938) described movable arms and attached musculature in larvae of the clypeasteroids, diamematids, and cidarioids, all of which have fenestrated spicules. I have never observed arm flaring in larvae of species that have simple spicules in arms homologous to the movable arms. The species of larvae with non-movable arms that I have observed are *Strongylocentrotus droebachiensis, S. franciscanus, S. purpuratus,* and *Toxopneustes roseus.* The four homologous arms of this last species contain a pair of simple and a pair of fenestrated spicules, but only the fenestrated pair is movable. This absence of arm movement has not been mentioned previously in the literature.

While it is possible that stiffer spicules and movable arms may prevent ingestion, no experimental work has been reported. Damage to surrounding tissues may occur even when larvae are not ingested whole.

In conclusion, forces generated in swimming do not appear to explain the correlation between arm length and fenestrated spicules. Predation is the next likely factor which might supply a mechanical rationale for skeletal variation.

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A MORPHOLOGICAL EXAMINATION OF SPERM-EGG INTERACTION IN THE FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII*

JOHN W. LYNN* AND WALLIS H. CLARK, JR.

Department of Animal Science, University of California, Davis, CA 95616

ABSTRACT

Eggs, covered by a bilayered investment composed of a 0.5 μm protein outer layer and a 2.5 μm mucopolysaccharide inner layer, are spawned through an externally held spermatophore following a female's ovigerous molt and mating. Mature sperm resemble everted umbrellas and consist of a cupped base with a single spike projecting from the convex surface. These sperm (<5/egg) attach base-first with the spike oriented perpendicularly to the investment. Within 15 seconds, the spike of the sperm bends at the base and contacts the investment. The spike penetrates the investment, the base is inverted, and fertilization occurs within two minutes. The spike remains briefly as a central core in the fertilization cone. The meiotic division of the egg resumes at this stage. First karyokinesis is completed approximately 6 hours post fertilization, but cytokinesis is suppressed until following the second karyokinesis at 8 hours postfertilization. First and second cytokinesis are simultaneous, and at 9 h the egg cleaves to the four cell stage.

INTRODUCTION

The sequence of morphological events leading ultimately to gamete fusion have been well documented in several vertebrate and invertebrate species (Austin, 1968; Epel and Vacquier, 1978, for review). In most of the gamete systems studied, motile male gametes are involved and follow the classic sea urchin fertilization model with minor modifications. Sperm approach the egg head first and encounter one or more investments. An acrosome reaction may occur at this time, allowing the sperm to recognize and bind to the vitelline layer. Cell fusion then occurs between the inner acrosomal membrane of the reacted sperm and the egg plasma membrane. This point of gamete fusion is often characterized by a fertilization cone.

In contrast, fertilization in the animal kingdom involving non-motile male gametes is poorly understood, particularly in the decapod crustaceans. Sperm of the decapods are considered atypical, nonmotile gametes and are separated into the multistellate sperm of the reptantians (crabs, crayfish, lobsters) and the unistellate sperm of the natantians (shrimp) (Wilson, 1928; Lu, 1976; Talbot and Summers, 1978). Although fertilization has been studied in both groups, the events that occur during reptantian fertilization are more extensively documented.

Early workers often reported observations of "exploded" or "everted" sperm in the reptantians (*e.g.*, Labbe, 1904; Koltzoff, 1906; Retzius, 1909; Binford, 1913; Fasten, 1924). Only a few of these early workers attempted to relate the sperm eversion process to a necessary step in fertilization (Koltzoff, 1906; Binford, 1913)

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* Present address: Dept. of Physiology and Biophysics, School of Medicine, P.O. Box 016430, University of Miami, Miami, FL 33101.

rather than a result of a simple change in osmotic pressure as suggested by other authors (Labbe, 1904; Koltzoff, 1906; Retzius, 1909). Subsequent investigators utilizing electron microscopy demonstrated that the explosive eversion of the sperm was involved in the penetration of the egg chorion (Brown, 1966; Hinsch, 1971; Goudeau, 1982). Sperm initially contact the egg with their stellate arms and bring the apical cap of the central cup of the sperm containing the acrosome and nucleus into direct contact with the egg investment. The sperm then "explosively everts" and penetrates the chorion to make contact with the oolemma. Both Hinsch (1971) and Brown (1966) have speculated that penetration of the egg chorion is at least partially facilitated by the release of lytic enzymes from the sperm. Actual membrane fusion between the gametes has not been observed.

Fertilization in natantians was erroneously described using light microscopy in a marine shrimp, *Penaeus japonicus* (Hudinaga, 1942). More recently sperm-egg orientation has been described at both the light and electron microscopy level in the shrimp *Sicyonia ingentis* (Clark, *et al.*, 1981a). These authors document an initial "spike first" orientation necessary for egg recognition and essential for the subsequent acrosome reaction to be effective in initiating sperm-egg fusion. As the acrosome reaction starts, the spike apparently depolymerizes, drawing the sperm into close association with the egg surface (Clark *et al.*, 1981a). The acrosome reaction proceeds (Yudin *et al.*, 1979; Clark *et al.*, 1981a), with resultant gamete binding (Yudin *et al.*, 1980; Clark *et al.*, 1981a). Similar observations on sperm-egg orientation and gamete activation have been noted in *Penaeus setiferus* and *P. aztecus* (Clark *et al.*, 1980). This sperm-egg orientation appears to be typical of the family Penaeidae (broadcast spawners).

Although Chow *et al.* (1982) briefly report sperm penetration of the egg of *Macrobrachium rosenbergii*, a complete description of sperm-egg interaction is lacking in the family Palaemonidae (shrimp which brood). Sperm of the palaemonid shrimp are often described as a "thumb tack" or "everted umbrella" shaped cell and consist of a cup-shaped main body with a single appendage (spike) (Nath, 1937; Brown, 1967; Pochon-Masson, 1968; Koehler, 1979; Clark *et al.*, 1980; Sandifer and Lynn, 1981; Chow *et al.*, 1982). An acrosomal region has not been clearly defined in the palaemonid sperm, although Koehler (1979) speculates a perinuclear veiscular region may function in an acrosomal capacity. In contrast, Pochon-Mason (1968, 1969) refers to the single spike as the acrosome and percutor organ. An explosive acrosome reaction as in the reptantians (Brown, 1966; Talbot and Summers, 1978) or elaborate acrosome reaction as in the penaeid shrimp, *S. igentis*,(Yudin *et al.*, 1979; Clark *et al.*, 1981a) is not inducible or observed in the sperm of the palaemonid shrimp (Lynn, unpublished).

Sandifer and Lynn (1981) have reported that the mature egg of *M. rosenbergii* is surrounded by an investment layer 3–5 μm thick and can be divided into two histochemically distinct regions; a proteinaceous, thin outer layer and a PAS positive inner layer. Orientation of the sperm to the egg and subsequent events leading to penetration of this formidable egg investment have thus far only been speculated on (Brown, 1967; Koehler, 1979; Sandifer and Lynn, 1981; Chow *et al.*, 1982). In the present paper the early events of sperm-egg interaction leading ultimately to gamete fusion are examined.

Materials and Methods

Male and female *M. rosenbergii* were obtained from the Institute of Marine Resources, Charleston, South Carolina. These animals are the Anuenue strain originally brought from Hawaii. Animals were held individually in ten gallon aquaria at 25°C. Females which had undergone an ovigerous molt were mated immediately with a male and checked after several hours for the presence of a spermatophore (Sandifer and Lynn, 1981). When females had successfully mated and spermatophores were present, the males were removed from the aquarium and the females were observed continuously until spawning.

Collection of spawned, fertilized eggs was facilitated by removal of the endopodites and exopodites of the second pair of pleopods allowing the fertilized eggs to fall to the aquarium bottom. Eggs were sampled at 15 second intervals post spawning with a large bore pipet for the first fifteen minutes after the initiation of spawning. For light microscopy, samples were fixed 8–10 hours in 4% glutaraldehyde with 3% sucrose in a 0.1 M PO_4 buffer at pH 7.4. The eggs were postfixed one hour in 1% osmium tetroxide, dehydrated in a graded acetone series, and embedded in a low viscosity epoxy resin (Spurr, 1969). Thick sections were cut with glass knives on a Sorvall MT-2B ultramicrotome and stained with borate buffered 0.5% toluidine blue at pH 11.0.

For scanning electron microscopy, samples were collected and fixed as above, dehydrated in a graded acetone series, critically point dried in CO_2, and scattered on SEM studs. Eggs were then sputter coated with 25 nm gold in a Polaron sputter coater and observed in a Philips SEM 501 scanning electron microscope at an accelerating voltage of 10–15 kV.

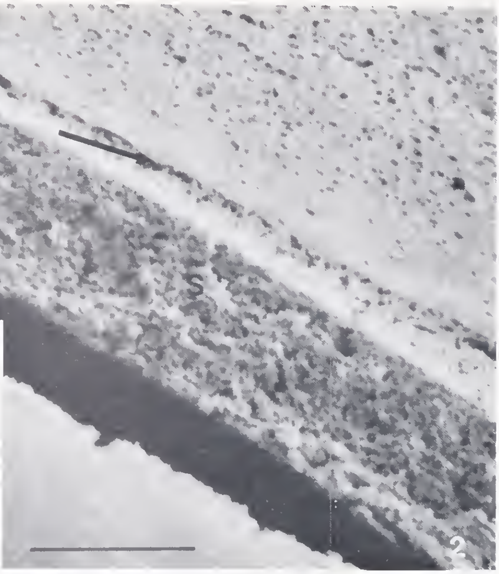
RESULTS

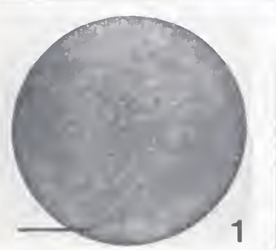
Fertilization in *M. rosenbergii* is external, occurring as the eggs are released from the gonopores at the base of the third pair of pereiopods and pass posteriorly over the spermatophore mass. The pleopods and thoracic coxae are positioned to form an enclosed channel for the eggs which ensures egg contact with the spermatophore mass.

Mature eggs removed from the ovary of an ovigerous female are approximately 500 μm in diameter and densely packed with yolk (Fig. 1). A germinal vesicle is not present and a single 3–5 μm investment surrounds the egg at this time. The investment layer may be resolved into a thin 0.5 μm porous outer layer and a much thicker 2.5 μm spongy inner layer (Fig. 2). Structures resembling micropyles are not observed in the thin outer layer when examined in thin plastic sections or with SEM.

Fertilized eggs collected immediately after spawning (0 time) generally have three to five sperm attached (Fig. 3). Sperm are oriented with their bases in contact with the investment and the spike erect (Fig. 4, 5). Numerous filamentous strands apparently originating from the base of the sperm, are intimately associated with the egg investment (Fig. 4). Cross sections of the sperm reveal the marked cupped appearance of the base as it sits on the investment resulting in contact being primarily restricted to the periphery of the sperm base (Fig. 5). During the next two minutes post spawning, a series of events involving changes in sperm orientation and penetration of the investment occurs and terminates with the incorporation of the sperm into the egg.

Fifteen to 30 seconds post spawning, the spike of the sperm begins to bend until the tip makes contact with the egg investment (Figs. 6, 7, 8). This bending characteristically occurs at the base of the spike. Cross sections of the sperm show the prominent cup shape of the base has been lost and a more intimate contact between the entire sperm base and the investment is achieved (Fig. 7). The filamentous extensions of the sperm base are still closely associated with the egg investment.





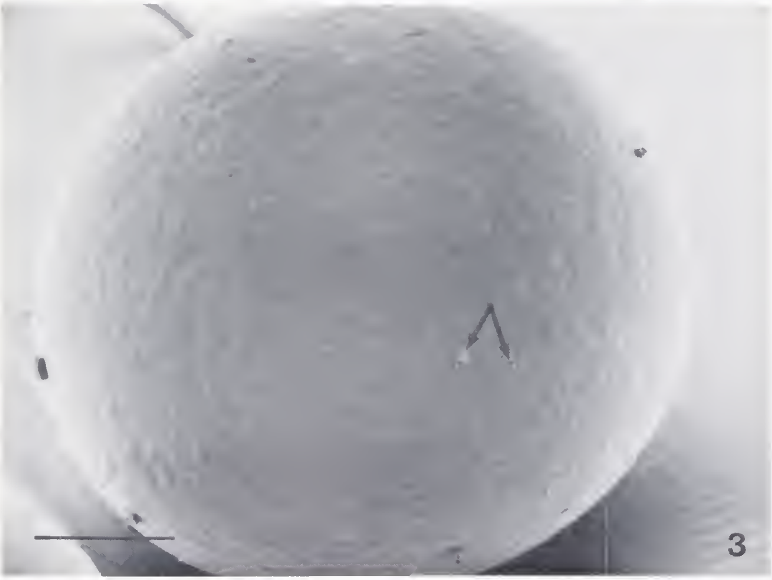
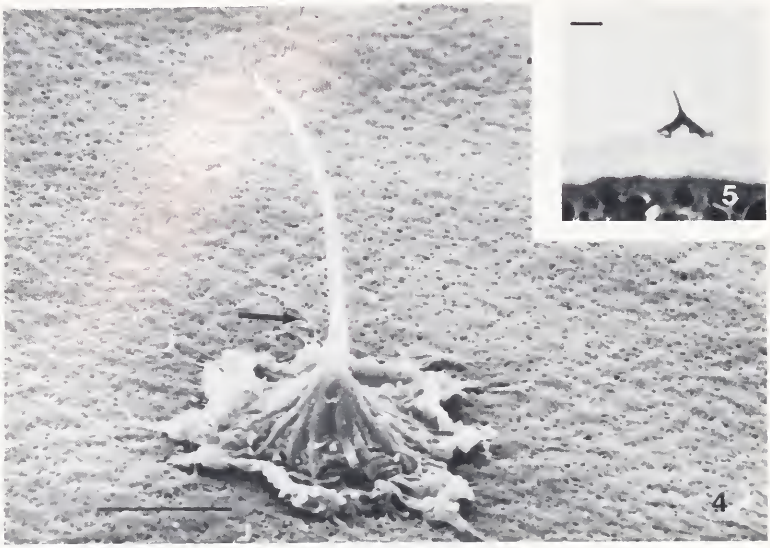


FIGURE 1. A mature *Macrobrachium rosenbergii* egg is approximately 0.5 mm in diameter and has a homogenous cytoplasm when viewed with phase microscopy. Bar = 200 μm.

FIGURE 2. The egg is covered by a bilayered investment and consists of a thin outer layer (arrow) which appears porous in scanning electron microscopy and a much thicker spongy layer (S) which remains closely opposed to the egg before fertilization. Bar = 5 μm.

FIGURE 3. The external surface of the egg investment has no differentiated area such as a micropyle, and following insemination, only 2–5 sperm (arrows) attach to the surface. Bar = 100 μm.

A breach in the outermost layer of the investment around the tip of the spike begins to appear by 45 seconds (Fig. 9). At this time, the base of the sperm partially dissociates from the egg investment and the edge distal to the point of contact lifts



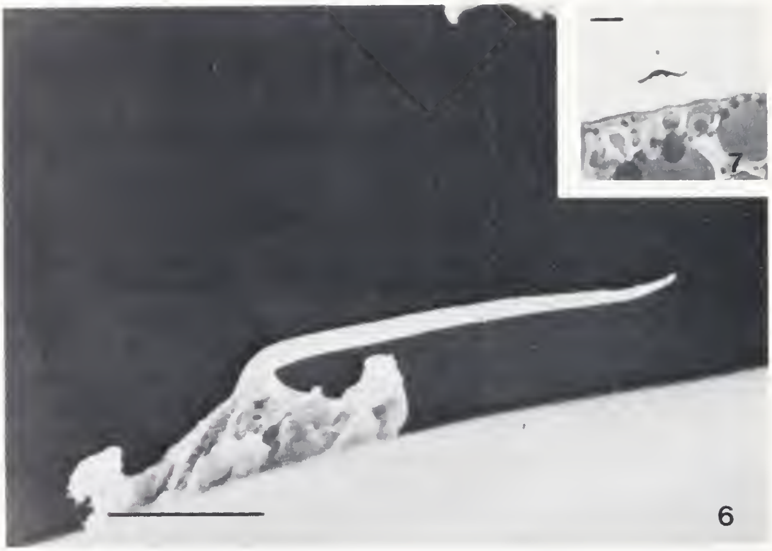


FIGURE 4. Mature sperm attach to the egg surface base first at fifteen seconds postspawning. The spike is diametrically opposed to the egg surface and filamentous strands from the sperm are associated with the egg investment (arrow). Bar = 5 μm.

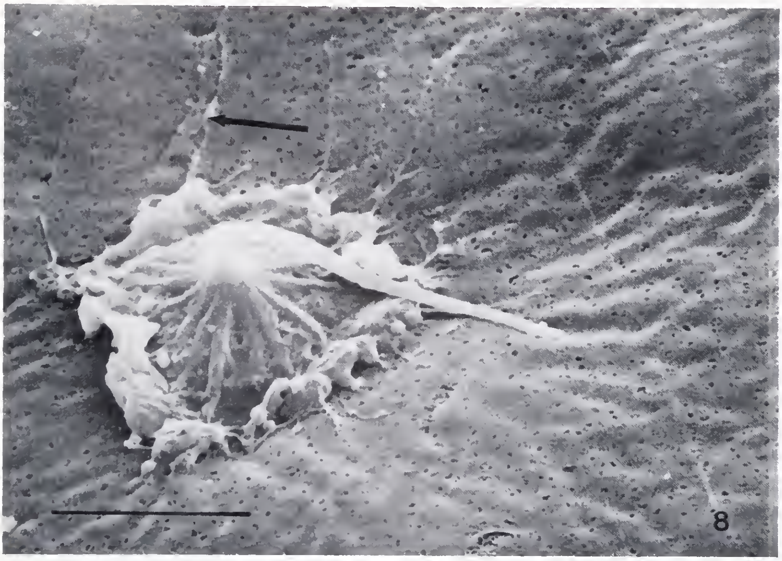
FIGURE 5. In thick plastic sections stained for light microscopy, the sperm base retains a marked cupped shape. Bar = 5 μm.

FIGURE 6. By fifteen to 30 seconds postspawning, the sperm spike bends close to the base. Bar = 5 μm.

FIGURE 7. In cross sections, the sperm base has noticeably flattened during the spike bending. Bar = 5 μm.

off of the investment surface (Figs. 9, 10). Filamentous extensions are no longer apparent in the disassociated region of the base (Fig. 10). Any evidence of former attachment in terms of investment scarring is not apparent.

By the end of 60 seconds, the spike is entering a breach in the investment (Fig. 11). At this time, the base of the sperm is almost entirely dissociated from the



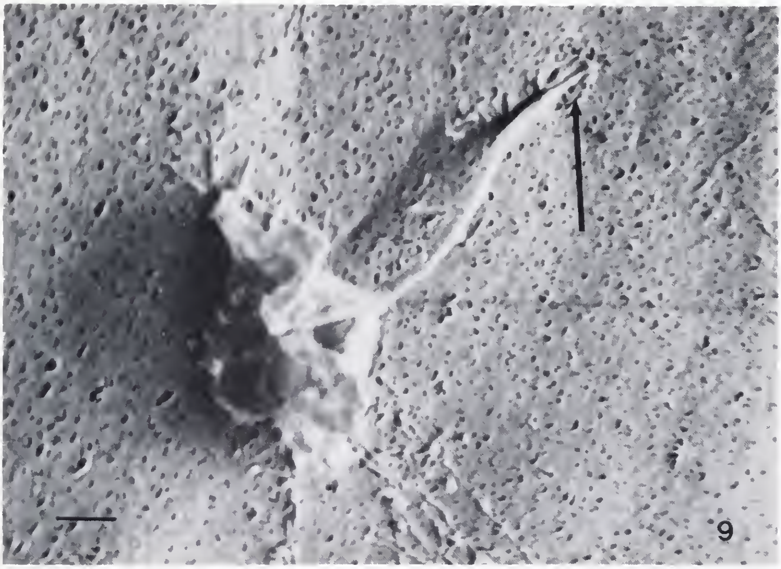
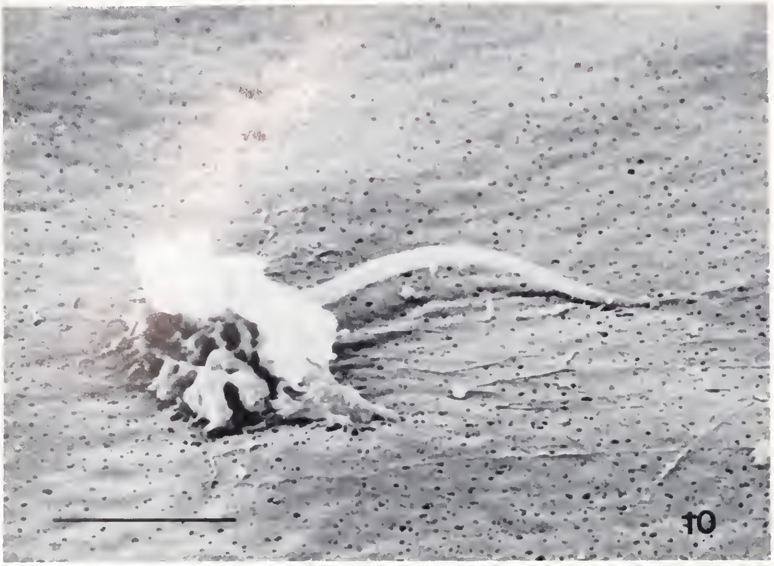


FIGURE 8. At the end of 30 seconds following spawning, the spike has contacted the egg investment. The base remains flattened, and filamentous material is still associated with the egg surface (arrow). Bar = 5 μm.

FIGURE 9. A breach in the investment surface begins to appear around the tip of the spike (arrow) by 45 seconds and the base of the sperm distal to the point of spike egg contact is detached from the egg surface. Bar = 1μm.

investment and in subsequent stages is entirely lifted off (Fig. 12). Some residual filamentous material may still link the base with the investment (Fig. 11). As noted earlier, no scarring of the investment is discernible where the base had originally contacted the investment.

Fusion has occurred by 90 seconds post spawning (Fig. 13, 14) and a fertilization cone is apparent. A dense core, perhaps representing elements of the spike, can be distinguished in the cone and is surrounded by densely staining cytoplasm (Fig. 14).



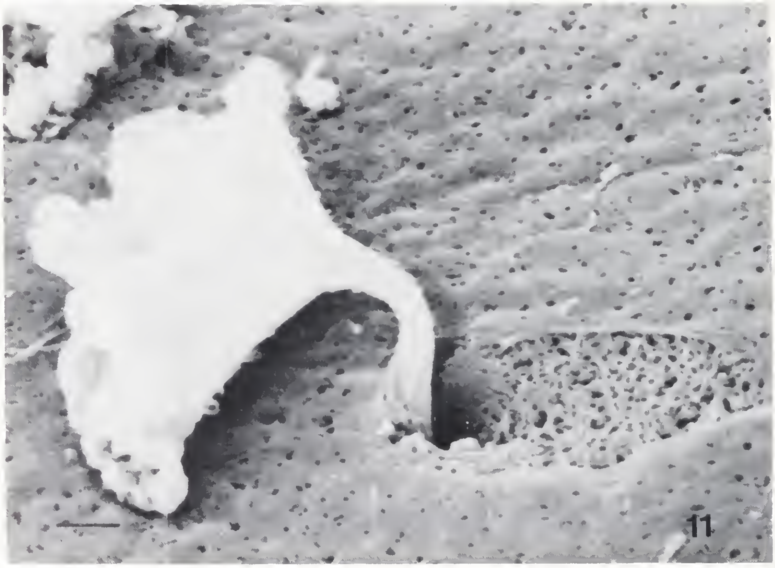
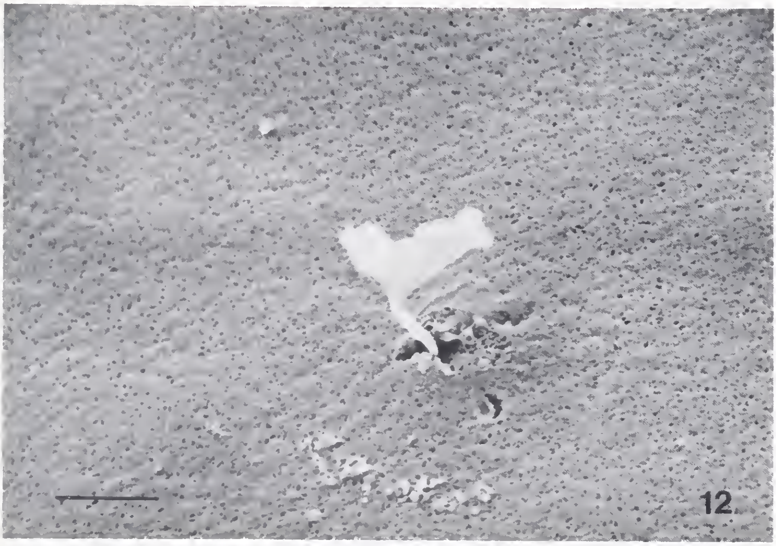
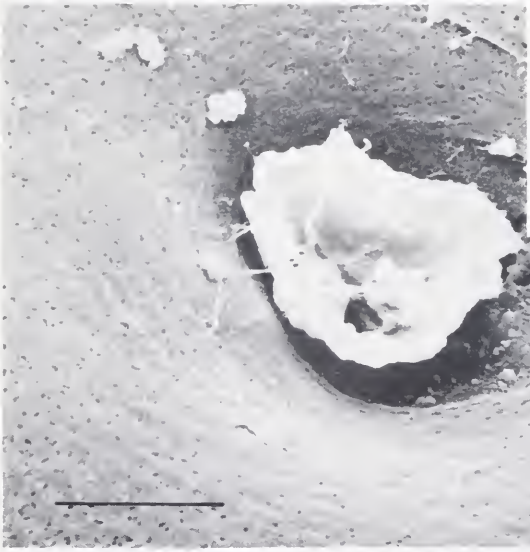


FIGURE 10. Filamentous material is now only associated with the egg at the point of contact of the sperm base. No scarring in the vicinity of the original attachment of the sperm base is visible. Bar = 5 μm.

FIGURE 11. Penetration of the spike through the egg investment continues and the breach in the investment surface enlarges. The cupped shape of the sperm base has returned by 60 seconds. Bar = 1 μm.

At this stage, the sperm base, now inverted from its original orientation to the egg, is in close association with the fertilization cone and lies within a funnel-shaped breach in the investment (Fig. 13, 14). The breach in the investment continues to enlarge, the fertilization cone diminishes in size and only remnants of the sperm base are apparent (Fig. 15, 16). Two minutes post spawning, a male pronucleus is





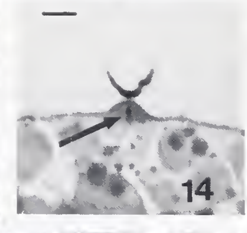
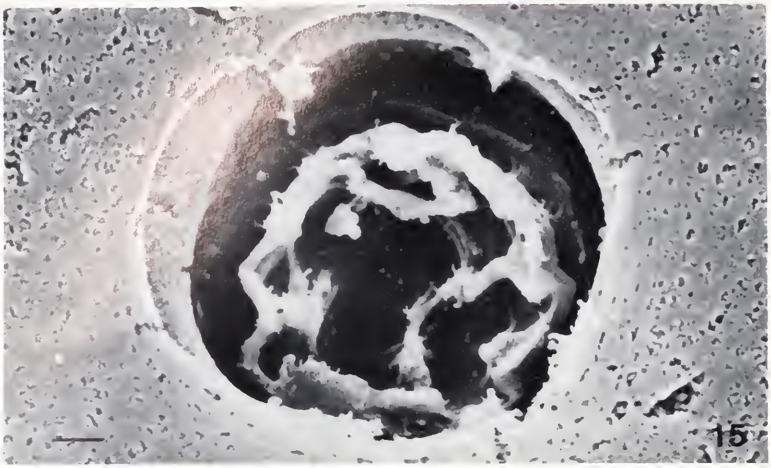


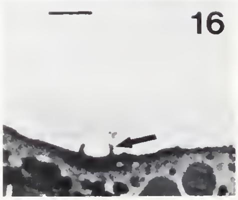
FIGURE 12. Rapidly the sperm base is lifted off the egg surface and is inverted over the spike and the breach in the investment surface. Notice that there is no additional scarring of the investment except in the immediate vicinity of the sperm spike. Filamentous material associated with the sperm base and the investment are now almost completely absent. Bar = 5 μm.

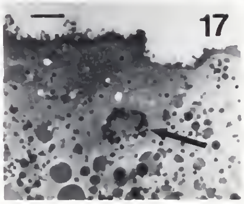
FIGURE 13. By the end of 90 seconds, the base of the sperm rests in a funnel shaped depression in the investment. The breach in the thin outer layer now exceeds the diameter of the sperm base. Bar = 5 μm.

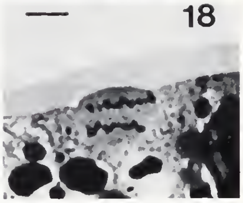
FIGURE 14. At the light level, the formation of a fertilization cone is observed and the spike of the sperm remains briefly as a dense central core (arrow). Note the tapered funnel shape of the investment in the area of the sperm base. Bar = 5 μm.

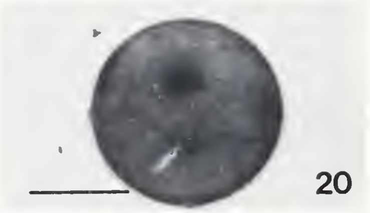
formed (Fig. 17). Meiotic divisions of the egg resume during the latter stages of sperm fusion, and meiotic figures are often observed at 5 to 10 minutes post fertilization (Fig. 18). Although monospermic fertilization appears to be the rule, in-

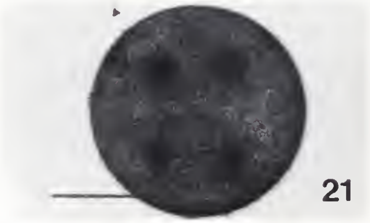












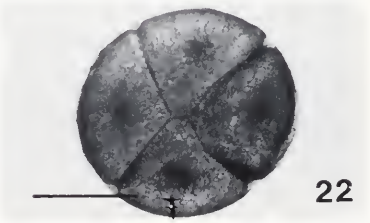


FIGURE 15. Within two to three minutes following spawning, the sperm has been incorporated into the egg and only remnants of the sperm base are visible with scanning electron microscopy. The breach in the investment surface will remain visible for at least twenty minutes post insemination. Bar = 1 μm.

FIGURE 16. The sperm base is often visible as a cup shaped remnant (arrow) at the surface of the plasmalemma at this stage, and the forming sperm pronucleus is visible as a thin dark band immediately beneath the base. Bar = 10 μm.

FIGURE 17. By 3–5 minutes, the point of sperm entry into the egg is identifiable only as a depression in the plasmalemma. The sperm pronucleus (arrow) has now begun to move into the egg cytoplasm. Bar = 10μm.

FIGURE 18. Concurrent with the penetration stage, the egg resumes its meiotic division and meiotic figures such as this one arrested during anaphase are often observed. Bar = 5 μm.

FIGURE 19. Four hours after spawning the zygote nucleus is centered in the egg cytoplasm. Bar = 250 μm.

stances of multispermic penetration were observed. In this study, only one in 500 eggs examined was polyspermic. Evidence of sperm penetration through the investment persists and may be observed up to 20 minutes post spawning.

At 28°C the nucleus initially appears at about 4 hours as a central dark stellate body in the central egg cytoplasm (Fig. 19), and the first mitotic karyokinesis occurs 6 hours post spawning (Fig. 20). Cytokinesis does not occur at this time and 2.5 to 3.0 hours after first karyokinesis, a second nuclear division produces four dense nucleoplasmic regions in the cytoplasm (Fig. 21). Approximately two hours after the second nuclear division (ten hours post spawning) the first and second cleavage divisions occur simultaneously resulting in four equally sized blastomeres (Fig. 22). Subsequent nuclear and cytoplasmic divisions occur in normal sequence.

Discussion

Two unique features characterize the sperm egg interaction observed in *M. rosenbergii.* First, there is a dramatic orientation-reorientation of the sperm following initial sperm-egg contact. Second, sperm of *M. rosenbergii* do not undergo an acrosome eversion as seen in the reptantians (Binford, 1913; Brown, 1966; Hinsch, 1971; Summers, 1978; Chanmanon, 1980), or an obvious acrosome reaction as observed in the natantian group Penaeidea (Yudin *et al.,* 1979; Clark *et al.,* 1981a, b).

In the present paper, it has been demonstrated that initial sperm-egg contact involves a base first orientation of the sperm to the egg investment. Koehler (1979) and Brown (1967) suggested such a base first orientation with the spike diametrically opposed in the caridean shrimp. This configuration seemed logical, since PAS positive vesicles were reported in the perinuclear region of the base by Koehler (1979) who suggested that they might serve as an acrosome. Brown (1967) has suggested a similar configuration based on preliminary observations on attempted *in vitro* fertilizations with the gametes of *Palaemonetes paludosus.* These authors never observed incorporation of the sperm into the egg, however. Chow *et al.* (1982) proposed that the sperm-egg interaction is based on a spike first approach based on light microscopic observations of sperm penetration into the egg. Early sperm egg interaction has not been reported, however in the caridean shrimp until the present paper. A similar reorientation of a sperm, once apparent sperm-egg binding has occurred, has never been reported in any animal system.

It is interesting to note that Yudin *et al.* (1980; see also, Clark *et al.,* 1981a) have demonstrated that successful sperm-egg contact in the Penaeidae is dependent on a spike-first orientation and is essential in *S. ingentis* for recognition binding to occur. In the case of the Penaeidea shrimp, it has been proposed that the depolymerization of the spike draws the sperm into more intimate contact with the egg (Kleve *et al.,* 1980; Yudin *et al.,* 1980; Clark *et al.,* 1981a) prior to the final stages of sperm-egg fusion 30 to 40 minutes later (Clark *et al.,* 1981a).

Sperm-egg binding after initial contact has been studied in several invertebrate groups (Vacquier and Moy, 1977; Brandiff *et al.,* 1978; Yudin *et al.,* 1980; Clark *et al.,* 1981a). In shrimp, oysters, and echinoderm systems, a sperm acrosome reaction occurs in close association with the egg and binding results. Although no

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FIGURE 20. At approximately 6 hours the first karyokinesis occurs but cytokinesis is suppressed. Bar = 250 μm.

FIGURE 21. Approximately 9 hours post spawning, the second karyokinesis takes place. Bar = 250 μm.

FIGURE 22. After the formation of the four nuclei, first and second cytokinesis occur simultaneously 2–3 hours later and results in a four cell stage. Bar = 250 μm.

acrosome reaction is observed in *M. rosenbergii* sperm, numerous filaments appear to originate from the sperm base. These filamentous elements, possibly pseudopodial in nature, may be involved in a recognition and/or binding phenomenon. These filamentous extensions possibly originate from the vesicular region observed by some authors in the sperm base (Brown, 1967; Koehler, 1979). Additional studies are required, however, to confirm this hypothesis.

Reorientation of the sperm, once initial sperm-egg contact has been established, involves a spike-bending phenomenon. Bending invariably occurs close to the base of the sperm though additional bends are occasionally seen. Movement of the spike in *M. rosenbergii* may either be a passive event (osmotic change) or an active event. A change in osmotic pressure would allow the spike to become less turgid and basically collapse onto the egg surface. Since fertilization is external and in freshwater, a provision for the active movement of either water or ions (activation of channels or pumps) would have to be present to permit the lowering of osmotic (turgor) pressure within the spike. Such a mechanism would suggest a gradual random collapse of the spike. A nondirectional collapse of the spike onto the egg surface does not appear to occur, however. The spike consistently bends at the base, with a secondary bending of the spike occurring as the base is inverting over the breach in the investment.

As an alternative, an active contractile process is suggested by several lines of evidence. Numerous parallel 6 nm filaments, resembling microfilaments, are observed in the spike and radial fibrils of *M. rosenbergii* sperm (Lynn and Clark, 1983). In addition, the radial fibrils are associated with centrioles at the base of the spike and are similar in morphology to rootlets observed in the base of flagellated sperm (Kleve and Clark, 1980). Actin has been reported in the spike of Penaeidae shrimp (Brown *et al.*, 1976) and Clark *et al.* (1981a) suggest that this actin may play an active role in drawing the sperm closer to the egg after spike contact has occurred. In *M. rosenbergii* filaments could differentially contract and cause the spike to bend. A second contractile process may also occur when the base of the sperm is inverted over the spike just prior to the incorporation of the sperm into the egg. Preliminary investigations have been unsuccessful, however, in identifying an actin component in the sperm of *M. rosenbergii* (Lynn, unpublished).

An additional feature observed in the process of fertilization of *M. rosenbergii,* but not limited to the species, is the apparent enzymic digestion of a localized opening in the egg investment. Initially, activity is restricted to the spike tip. Enzymic activity associated with the passage of the sperm through extraoocytic layers has been reported in vertebrates (Yanagamachi and Teichman, 1972; Stambaugh, 1978) as well as invertebrates (Levine *et al.*, 1978; Green and Summers, 1980). In other animal sperm, the enzymic activity has been classically associated with the acrosome and its activation during the acrosome reaction (Stambaugh, 1976; Levine, 1978). Although an obvious acrosome reaction is not observed in *M. rosenbergii* sperm, an enzyme may be localized on/in the spike and released or activated upon contact with the egg investment. If a trypsin-like enzyme is involved as in other animal sperm (Levine *et al.*, 1978; Stambaugh, 1978), its presence could easily be assayed by inhibiting fertilization with known protease inhibitors.

In the present paper, two interesting features of gamete interaction in natantians have been demonstrated: first, a two stage orientation-reorientation of the sperm in contact with the egg, including bending of the sperm spike; and second, the penetration of the egg investments possibly involving lytic enzymes. Fertilization in *M. rosenbergii* may provide a new system for investigation of events such as the enzymic digestion of egg investments and types and functions of contractile systems in gametes. Studies on these phenomena have been initiated and will be reported in a later paper.

ACKNOWLEDGMENTS

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THE FINE STRUCTURE OF THE MATURE SPERM OF THE FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII*

JOHN W. LYNN* AND WALLIS H. CLARK, JR.

Department of Animal Science, University of California, Davis, California, 95616

ABSTRACT

Electroejaculated spermatophores from male *Macrobrachium rosenbergii* contain mature sperm which resemble everted umbrellas. The nonflagellated, nonmotile sperm consist of a 10 μm main body (base) and a single 12–15 μm spike extending from the convex surface of the base. The nucleus is flocculent (decondensed), contains basic proteins, is not delimited by a nuclear membrane, and is housed in the base. The cytoplasm of the convex base contains fifteen to twenty radial fibrils which anastomose to form the spike. Both the spike and the radial fibrils are cross-striated with a periodicity of 35 nm. These structures contain 6 nm filaments running perpendicularly to the cross-striations. A pair of centrioles composed of nine doublet tubules are located acentrically near the origin of the spike in the cytoplasm of the base. Radial fibrils are interconnected to the centriolar matrix by accessory fibrils. No clearly definable acrosomal region is identifiable morphologically or histochemically.

INTRODUCTION

Considered atypical due to their lack of a flagella and midpiece, decapod crustacean sperm attracted early investigators and prompted numerous light microscopic studies (*e.g.*, Labbe, 1903; Koltzoff, 1906; Retzius, 1909; Binford, 1913; Fasten, 1924; Bowen, 1925; Nath, 1937; Worley, 1939; McCroan, 1940). Often these early reports were directed at determining homologies between the nonflagellated crustacean sperm and the flagellated sperm of other animals. Later investigators have continued the morphological studies on the nonmotile decapod sperm using electron microscopy and extensive histochemical techniques (*e.g.*, Ruthman, 1958; Yasuzumi, 1960; Moses, 1961; Pochon-Masson, 1963, 1969; Brown, 1966; Hinsch, 1969; Talbot and Summers, 1978; Koehler, 1979). Morphological descriptions have resulted in the crustacean decapod gametes being divided into two classes, the unistellate sperm of the natantians (shrimps) (Clark *et al.*, 1973; Brown *et al.*, 1976; Lu, 1976; Talbot and Summers, 1978), and the multistellate sperm of the reptantians (lobsters, crabs, crayfish) (Moses, 1961; Brown, 1966; Hinsch, 1973; Talbot and Summers, 1978). These studies have shown the decapod sperm consists basically of a spherical main body with a variable number of radiating appendages (Wilson, 1928; Talbot and Summers, 1978). The main body is generally believed to house the nucleus, acrosome, and a lamellar region, though variations of this scheme have been documented (Yudin *et al.*, 1979).

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* Present address: Dept. of Physiology and Biophysics, School of Medicine, P. O. Box 016430, University of Miami, Miami, Florida 33101

Although numerous light and fine structural studies are available on the structure of the natantian sperm (Koltzoff, 1906; Retzius, 1909; Nath, 1937; Pochon-Masson, 1968b, 1969; Clark *et al.*, 1973; Koehler, 1979; Lu, 1979; Yudin *et al.*, 1979; Kleve *et al.*, 1980), natantian sperm are still poorly understood as compared to the reptantian sperm. Functions have been demonstrated for many of the structures in the reptantians (Brown, 1966; Hinsch, 1971; Talbot and Chanmanon, 1980; Goudeau, 1982) and in the Penaeidae natantians (Yudin *et al.*, 1979; Clark *et al.*, 1980). Brief reports on the morphology of the mature sperm of the Caridea natantians have illustrated the great diversity of sperm structures between the palaemonids and penaeids (Pochon-Masson, 1969; Lynn, 1981; Sandifer and Lynn, 1981; Sellos and Le Gal, 1981). As an integral part of fertilization studies in the freshwater caridean shrimp *Macrobrachium rosenbergii,* a thorough description of the mature sperm using electron microscopy and histochemical techniques is presented here.

MATERIALS AND METHODS

Male *M. rosenbergii* were obtained from the Institute of Marine Resources, Charleston, South Carolina, and held individually in compartmentalized 45 gallon fiberglass tanks. Aerated flow-through water was maintained at 25–28°C.

Spermatophores containing mature sperm were obtained from males by electroejaculation (Sandifer and Lynn, 1981). Isolated free sperm were obtained by homogenization of spermatophores in a 20% MBL sea water solution (Cavanaugh, 1956) with a pyrex ground glass tissue homogenizer (0.15 mm clearance). A subsequent 5–10 min incubation with 0.01% amylase or 0.01% diatase (Sigma) in a Tris buffer (pH 7.4) eliminated much of the acellular spermatophore contamination. This suspension was layered on a 10% sucrose solution and centrifuged at 2000 × g for 10 min. The resulting pellet was resuspended and washed four times with Tris buffer. Sperm were examined with phase contrast and differential interference phase contrast optics.

Whole sperm were analyzed histochemically using acridine orange, periodic acid-Schiff (PAS) reagent, Feulgen and basic fast green stains. For acridine orange, sperm suspensions were treated live (Thompson, 1966) and examined on an Olympus epiflourescent microscope. Sperm suspensions were also fixed in 0.1 *M* PO_4 buffered 10% formaldehyde at pH 7.4. These sperm suspensions were stained with PAS and Feulgen reagent (Thompson, 1966), basic fast green (Alfert and Geschwind, 1953), and acid fast green (Langreth, 1969).

For scanning electron microscopy (SEM), spermatophores were compressed between two coverslips and fixed in 4% glutaraldehyde buffered with 0.1 *M* PO_4 at pH 7.4 for 20 min. After postfixation, samples were rapidly dehydrated in a graded acetone series and critically point dried with CO_2. Samples were mounted on SEM studs and coated with 20 nm gold in a Polaron sputter coater with a rotating stage. Sperm were examined on a Philips SEM 501 microscope at an accelerating voltage of 10 kV.

For transmission electron microscopy (TEM), free sperm suspensions and spermatophores were fixed for two hours in a 0.1 *M* PO_4 buffered 4% glutaraldehyde solution containing 1% tannic acid at pH 7.4. Sperm were washed in 0.1 *M* PO_4 buffer, postfixed 30 min in 0.2 *M* PO_4 buffered 0.5% osmium tetroxide, washed again in PO_4 buffer, rapidly dehydrated in a graded acetone series, and embedded in a low viscosity epoxy resin (Spurr, 1969). Sections were cut with a diamond knife on a Sorvall MT-2B ultramicrotome, stained with saturated methanolic uranyl acetate and aequeous lead citrate (Venable and Coggeshall, 1965), and examined on a Hitachi HU11E electron microscope at an accelerating voltage of 75 kV.

RESULTS

Spermatophores

A single pair of spermatophores may be obtained from a mature male once a day using electroejaculation. As the spermatophores are extruded from the terminal ampullae through the gonopores at the base of the fifth pair of walking legs, the distal tips contact and the spermatophores join and adhere as they move posteriorly. After a brief period of hydration (10–30 min), the spermatophores from *M. rosenbergii* are easily differentiated into two morphologically distinct regions (Fig. 1). In the fused condition, two white opaque strips containing the mature sperm are observed on the lateral edges of the spermatophore complex. Proximal to the fused medial line are two thicker, more translucent regions. These regions contain no sperm and can be mechanically removed to reduce the cellular and acellular contamination they otherwise contribute to sperm suspensions. The remaining spermatophore matrix is PAS positive and is largely removed by treatment with amylase or diatase. Enzyme treatment does not appear detrimental to the sperm.

General sperm morphology

Mature *M. rosenbergii* sperm isolated from the spermatophore mass are nonmotile and resemble an "everted umbrella" when observed with phase microscopy (Fig. 2). The main body (base) of the sperm is slightly cupped and a single appendage (spike) projects from the convex surface of the base. The peripheral edges of the sperm base have a ruffled or irregular appearance. Fourteen to twenty radial fibrils converge at the center of the base (Fig. 3). At the point of convergence and immediately beneath the spike is a highly birefringent locus.

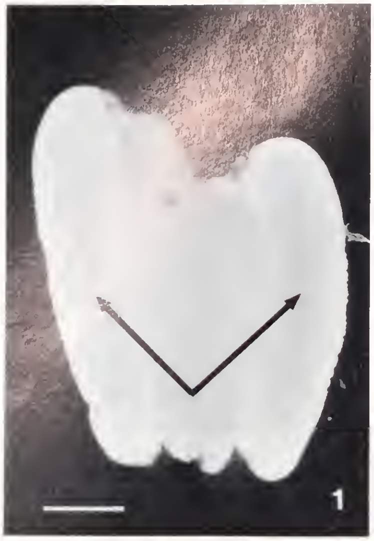
In SEM, the radial fibrils can be clearly seen anastomosing into the spike (Fig. 4). The radial fibrils have a diameter of 225 nm and the 12–15 μm long spike tapers from approximately 850 nm at the base to 350 nm at the tip. Slightly irregular in shape, the base of the sperm is 9–10 μm in diameter and approximately 4 μm in height (Fig. 4, 5). The sperm can be divided into three morphologically distinct regions when examined at the fine structural level (Fig. 6). These regions which will be discussed in the following order are the base, the spike, and the cap-like area on the convex surface of the base.

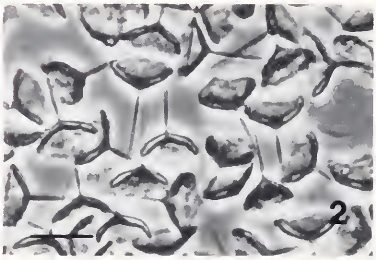
Morphology and cytochemistry of the main body

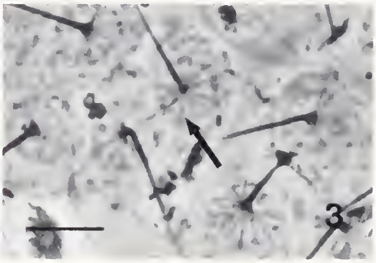
The base contains the nucleus, identified as a Feulgen-positive region. Sperm cells stained with acridine orange exhibit a green flourescence in the base, further indicating double stranded nucleic acids. Basic proteins are also associated with and confined to the nuclear region as indicated by basic fast green staining. With TEM, the nucleus appears as a flocculent, decondensed area (Fig. 6) and almost entirely fills the cup-shaped base. The nucleus is not limited by a nuclear envelope, but rather by the plasmalemma on one side and the membrane bound region of the cap on the other. In tannic acid-glutaraldehyde fixations, the plasmalemma around the nuclear cup is relatively smooth (Fig. 6).

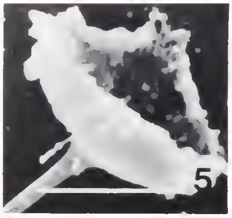
Spike morphology and cytochemistry

The spike of the sperm is composed primarily of protein as indicated by positive acid fast green staining and digestion of the spike by trypsin. This region of the cell does not exhibit a positive reaction to PAS.









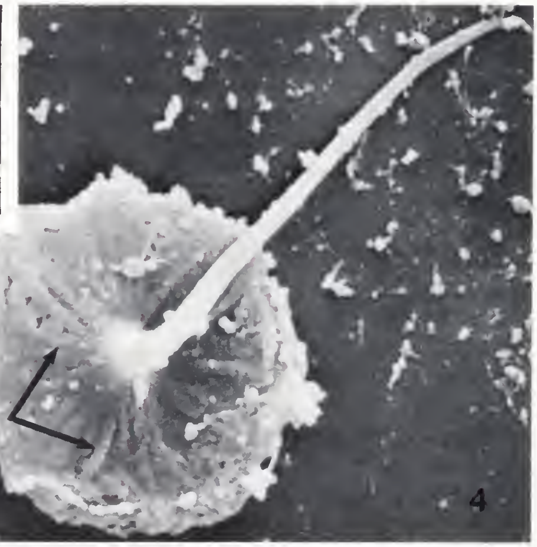


FIGURE 1. Fused electroejaculated spermatophore pair showing medial translucent region and the lateral opaque region (arrows) which normally contain the mature sperm. Bar = 1 mm.

FIGURE 2. A phase micrograph of mature *Macrobrachium rosenbergii* sperm showing the normal everted umbrella configuration. Bar = 10 μm.

FIGURE 3. A phase micrograph of mature sperm showing the radial fibrils (arrow) in the sperm base which anastomose to form the spike. Bar = 10 μm.

FIGURE 4. A scanning electron micrograph more clearly illustrating the anastomosing radial fibrils of the base. Bar = 5 μm.

FIGURE 5. The distinct cup shape of the base with the spike projecting from the convex surface is shown. Bar = 5 μm.

Viewed with TEM, the spike is continuous with the radial fibrils of the cap (Fig. 6). At high magnifications, the spike has a cross striated appearance, the striations running perpendicular to the length of the spike (Fig. 7). The cross striations have

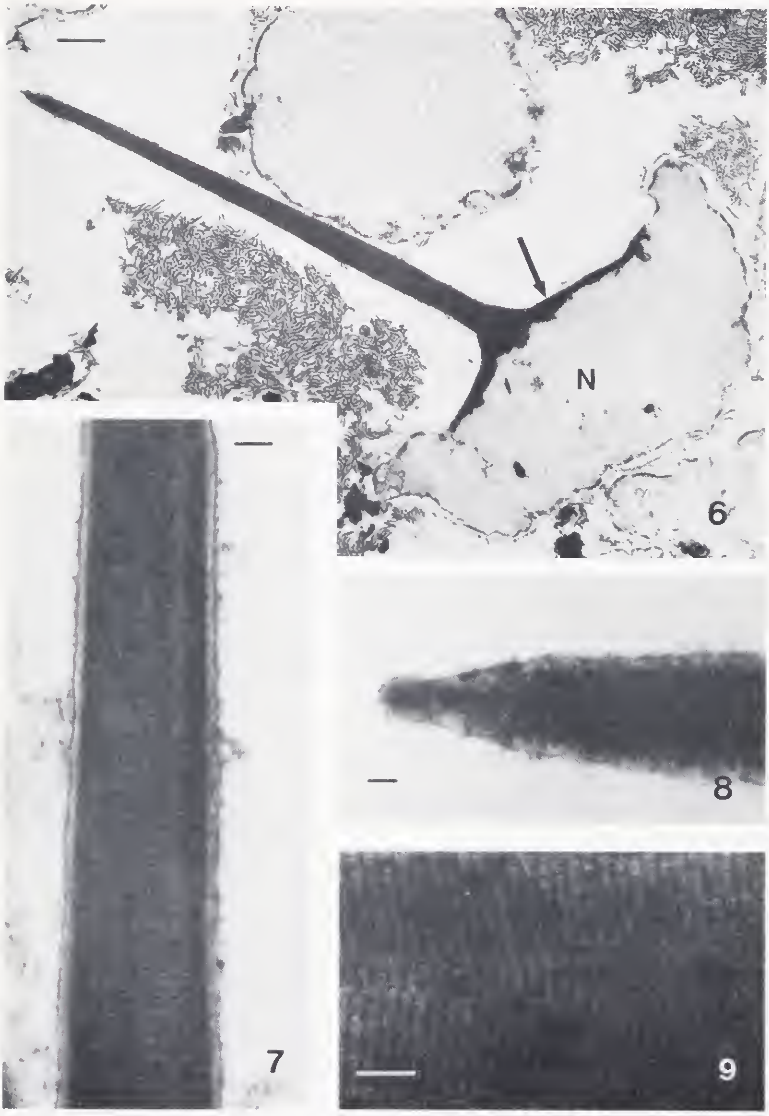


FIGURE 6. At the fine structural level, a longitudinal section through a mature sperm shows the decondensed nuclear region (N) in the base, the caplike region (arrow) housing the radial fibrils, and the cross-striated spike. Bar = 1 μm.

FIGURE 7. The cross striated appearance of the spike is apparent at higher magnifications. Note the numerous 6 nm filaments running perpendicular to the cross-striations. Bar = 0.1 μm.

FIGURE 8. The spike terminates in a 50 nm bulbous tip. Bar = 0.1 μm.

FIGURE 9. Occasionally the cross striations of the spike are observed to be out of register, indicative of the anastomosing radial fibrils of the cap-like region. Bar = 0.1 μm.

a 35 ± 2.5 nm periodicity. Numerous 4–7 nm filaments run longitudinally between the dense bands of the cross striations and both filaments and cross striations run the length of the spike terminating in a 50 nm spike tip (Fig. 8). The cross striations of the spike are occasionally out of register (Fig. 9), indicative of the anastomosing radial fibrils from the cap.

Oblique sections close to the base illustrate the anastomosing fibrils (Fig. 10). Although striations are often apparent in the oblique sections, the periodicity may appear compressed due to the angle of section. Sections through the spike midway between the tip and the base are often amorphous and structural elements are difficult to resolve. Closer to the tip, however, tubule-like structures with an outside diameter of 26 nm are observed (Fig. 11). Such microtubular structures are observed only in cross sections at or near the tip of the spike.

Cap region morphology and cytochemistry

Fifteen to twenty radial fibrils that anastomose to form the sperm spike comprise the major substructure of the cap (Figs. 6, 12). The cap region demonstrates a weak, nonspecific reaction to PAS. The fibrils react positively with acid fast green indicating a proteinaceous nature. When viewed at the fine structural level, the radial fibrils are continuous with the spike and have the identical, periodic cross striation and 4–7 nm filaments (Fig. 12).

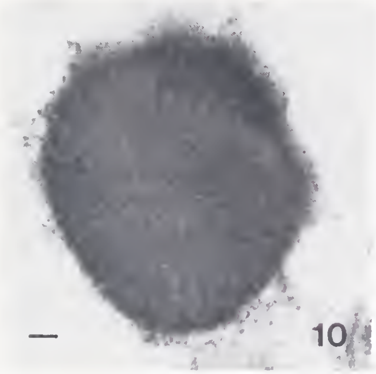
The cap region, including a thin band of cytoplasm, appears to be at least partially separated from the nucleus by a membrane which appears double in some areas (Fig. 13). Whether the membrane system limiting the cap is complete and fuses with the single plasmalemma surrounding the sperm is presently unclear. In the cytoplasmic band of the cap, a pair of centrioles are housed acentrically in relation to the base of the spike (Figs. 13, 14). The centriolar pair is embedded in a dense matrix. Dense bodies are observed in association with the centrioles and are apparently sections through the cross striated fibrils associated with the centriolar matrix (Fig. 16). These fibrils intersect this matrix independent of the centrioles' orientation (Figs. 13, 14) and extend between the centriolar matrix and the radial fibrils of the cap. Each centriole is approximately 160 nm in diameter and 225 nm in length consist of nine doublet microtubules (Fig. 15) with a pair of dynein-like arms attached to each doublet. A flocculent material occupies the central core and appears to have a spoke-like arrangement attached to each of the doublets.

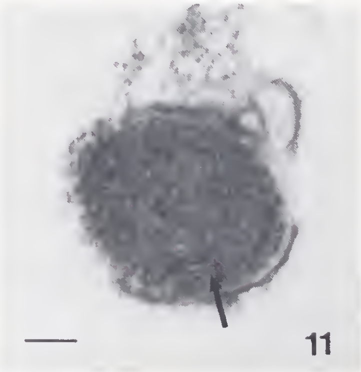
Discussion

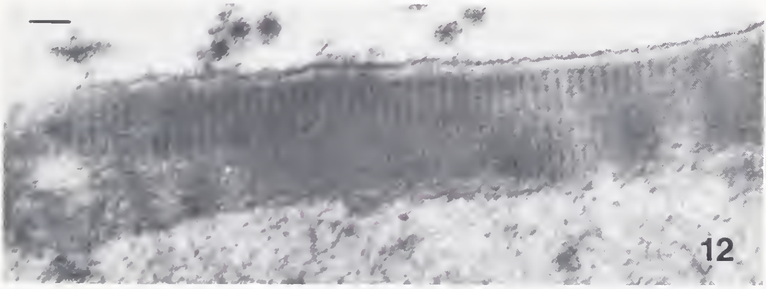
Although some fine structural differences are present, the sperm of *M. rosenbergii* conform to the basic morphology reported in other caridean shrimp (Nath, 1937; Pochon-Masson, 1968b, 1969; Koehler, 1979; Sellos and Le Gal, 1981). The mature sperm are nonmotile and comprise a cup-shaped base housing the nucleus and a single spike which projects from the convex surface of the base.

The nucleus is decondensed, as is typical of the decapod crustacean sperm, and a nuclear envelope is absent as in other natantians (Pochon-Masson, 1969; Clark *et al.*, 1973; Lu, 1976; Talbot and Summers, 1978; Kleve *et al.*, 1980; Goudeau, 1982). Basic proteins are associated with the nucleus in *Macrobrachium* sperm. Basic proteins have also been reported in other decapod sperm (Bloch, 1966; Vaughn, 1968; Koehler, 1979; Sellos and Le Gal, 1981) but may be absent in some cases (Chevaillier, 1966; Lu, 1976; Kleve *et al.*, 1980). The basic proteins observed in the sperm of the *Palaemon serratus* have been identified as histones with no protamines present (Sellos and Le Gal, 1981). The significance of the basic proteins in some decapod sperm but not in others is presently unclear.

The nucleus extends to the plasmalemma of the base with no intervening region of cytoplasm as shown in *P. serratus* (Sellos and Le Gal, 1981). It should be noted, however, that membranous blebbing and whorls are found associated with the plasmalemma when *M. rosenbergii* sperm are fixed with paraformaldehyde or glutar-







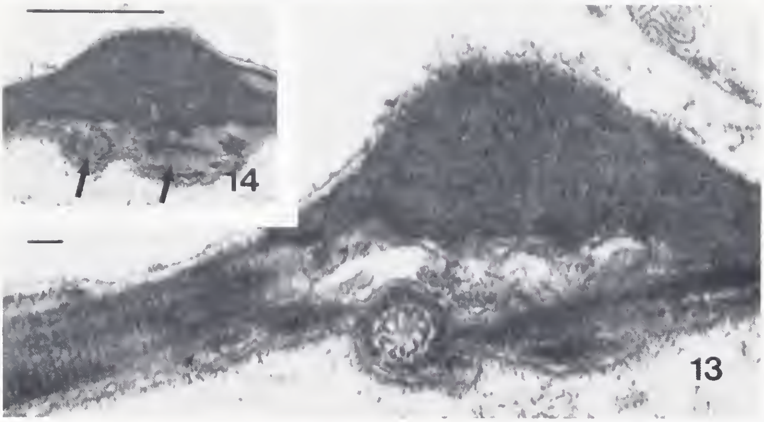


FIGURE 10. A cross section through the base of the sperm spike demonstrates the slightly compressed cross striation (due to the angle of the section) of the anastomosing radial fibrils. Bar = 0.1 μm.

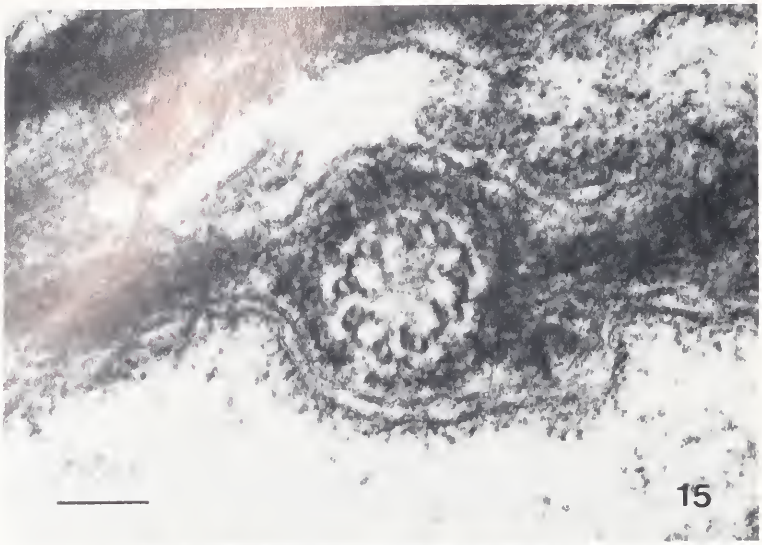
FIGURE 11. At the tip of the spike cross sections reveal microtubular structures (arrow) in cross section. Similar structures have not been observed in longitudinal sections. Bar = 0.1 μm.

FIGURE 12. The radial fibrils of the cap-like region of the base show the same cross-striated appearance and presence of 6 nm filaments as are present in the sperm spike. Bar = 0.1 μm.

FIGURE 13. Interconnecting fibrils between the radial fibrils of the base and the centriolar matrix are also observed in cross section of the sperm base and contain cross striations and filaments as observed in the spike and the radial fibrils. Bar = 0.1 μm.

FIGURE 14. Centrioles are located in the main body of the sperm (arrows) and as shown in this section are housed acentrically at the base of the spike. Bar = 1.0 μm.

aldehyde. These whorls appear to be equivalent to the perinuclear vesicular region described in the sperm of *Palaemonetes paludosus* (Brown, 1967; Koehler, 1979). Koehler (1979) identified a PAS positive reaction in this region of the sperm and



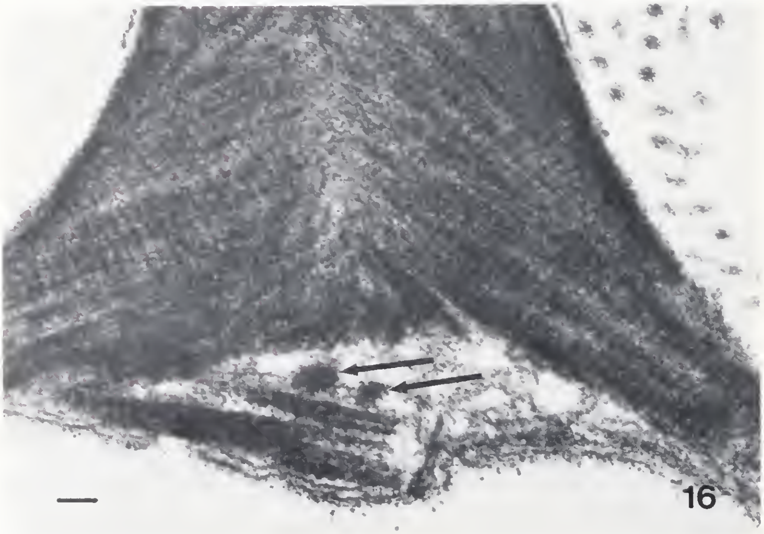


FIGURE 15. Nine doublet tubules comprise the centrioles as shown at high magnification in transmission electron micrographs. Notice the interconnecting fibrils intersecting the centriolar matrix. Bar = 0.1 μm.

FIGURE 16. A centriole in longitudinal section is often associated with dense bodies (arrows). These dense bodies probably represent cross sections through the interconnecting fibrils. Bar = 0.1 μm.

thus suggested it was acrosomal in nature. This suggestion is questionable, however, since fertilization studies have demonstrated the spike is the leading edge of the sperm during investment coat penetration (Lynn and Clark, 1983).

In the natantians, the spike has been suggested to be part of the acrosome (Pochon-Masson, 1969; Lu, 1976; Kleve *et al.*, 1980), a role also suggested for the cross striated structures associated with the isopod and schizopod sperm (Reger and Fain-Maurel, 1973) and the cross striated structure which forms a part of the acrosomal complex in the octopus (Galangau and Tuzet, 1968). Pochon-Masson (1969) refers to the *Palaemon elegans* sperm spike as the acrosome and percutor organ based on analogies to the reptantian sperm. No acrosome reaction or characteristic histochemical staining, however, was observed in *P. elegans.* The spike of *Sicyonia ingentis* and *Penaeus aztecus* sperm has been demonstrated to be a part of an elaborate acrosomal complex (Lu, 1976; Clark *et al.,* 1980; Kleve *et al.,* 1980; Clark *et al.,* 1981) based on observations both *in vitro* and during the fertilization process (Yudin *et al.,* 1979; Clark *et al.,* 1980; Kleve *et al.,* 1980; Clark *et al.,* 1981) and may represent the normal function of the spike in the penaeid shrimp sperm. The structure of the sperm spike in *M. rosenbergii* is dissimilar to the structure observed in *P. aztecus* and *S. ingentis,* however, and its possible role as an acrosome is still uncertain since an acrosomal reaction has not been observed in the Macrobrachium cell (Lynn, 1981), although a dramatic spike bending process is observed during fertilization (Lynn and Clark, 1983).

The cross striated appearance of the spike and the radial fibrils, the numerous 6 nm filaments which run the length of these structures, and the unique spike bending process involved in fertilization clearly define the spike complex as the most unusual feature of the *M. rosenbergii* sperm. In agreement with other authors (Lu, 1976; Talbot and Summers, 1978), the present study suggests that the spike of the caridean sperm is not analogous to the spikes of the reptantian sperm, since *M. rosenbergii* sperm appendages contain neither nuclear material nor extensive microtubular complexes. Although microtubules may be present in cross sections of the tip of macrobrachium sperm spikes, they are not observed in longitudinal sections and certainly do not represent as prominent a feature as in some reptantian species.

Cross-striations similar to those observed in the spikes of Macrobrachium sperm have also been reported in the sperm of *P. elegans* (Pochon-Masson, 1969) and *P. paludosus* (Koehler, 1979; Brown, 1967). In contrast to the 35 nm periodicity in *M. rosenbergii* sperm, both Koehler and Pochon-Masson report the periodicities of the striations as 22–26 nm. All three species, however, do contain 4–6 nm filaments running parallel to the length of the spike. Similar filaments are also observed in the radial fibrils and resemble microfilaments. Currently the function and biochemical composition of the spike other than its proteinaceous nature is uncertain.

It is interesting that centrioles are not observed at the base of the spike in the *P. aztecus* and *S. ingentis* sperm, whereas a pair of centrioles are closely associated with the base of the spike and radial fibrils in Macrobrachium sperm. In contrast to a typical centriole, however, centrioles in Macrobrachium sperm consist of nine doublets with dynein-like arms and no central axis structures. By comparison, a typical centriole is composed of nine triplets, occasionally with internal axis spokes (McNitt, 1974). Koehler (1979) reports no centriolar structures in the mature sperm of *P. paludosus,* although centrioles are observed in *P. elegans* and *Crangon vulgaris* (Pochon-Masson, 1968a, 1969). Similar unusual centrioles have been demonstrated in the sperm of *Eupagurus bernhardus* (Pochon-Masson, 1963), several species of isopod sperm (Cotelli and Lanzavecchia, 1972; Cotelli *et al.,* 1975), and in somatic cells (Hoage and Kessel, 1968). Although no specific function has been attributed to these centrioles, they may act as control centers for contractile processes (Salisbury and Floyd, 1978; Kleve and Clark, 1980).

Two structures which have been observed in association with the centrioles of *M. rosenbergii* are intersecting fibrils and "dense bodies" resembling satellites. The actual identity of the latter is uncertain since no microtubules are associated with them and they are located closer to the centriole than is normal for satellites (Kleve and Clark, 1980). It would appear that these "dense bodies" are actually sections of fibrils intersecting the centriolar matrix. These fibrils contain the same striated pattern and periodicity as is seen in the spike and radial fibrils. The association with the centrioles and their morphology are suggestive of ciliary rootlets. Although Fawcett and Porter (1954) report considerable variation in the periodicity of ciliary rootlets, it is usually greater than that seen in the Macrobrachium sperm structure.

Three functions have been suggested for centrioles and their associated structures in flagellate sperm; an organization center for the production of the sperm flagellum; as a division center after incorporation of the sperm into the egg at fertilization (Monroy, 1965); and, as a contractile center for altering sperm symmetry (Kleve and Clark, 1980). The presence of centrioles in a non-flagellated sperm poses a question as to their function. Obviously, they do not act as a polymerization center for flagella since none are present. It might be speculated that they are utilized as a division center in the egg after fertilization as has been suggested for a number of flagellated sperm (Monroy, 1965). Centrioles are absent, however, in several crustacean sperm (Yasuzumi, 1960; Chevaillier, 1966; Koehler, 1979). Finally, the arrangement of the centrioles and accessory fibrils from the radial fibrils might be indicative of contractile ability. The morphology of the spike and fibrils are reminiscent of ciliary rootlets, and numerous 6 nm filaments resembling microfilaments are present within the spike and fibrils. Although other sperm are reported to contain actin microfilaments (Tilney *et al.*, 1973; Brown, *et al.*, 1976) current electrophoretic and immunological evidence suggests actin is not present in *M. rosenbergii* sperm (Lynn, 1981). The question of contractile ability is still intriguing, however, for two reasons. First, the fibrils do resemble centriolar rootlets. Second, while the above functions were attributed to actin, there is increasing evidence suggesting non-actin proteins may also be involved in cell movement (Klass and Hirsch, 1981).

Obviously, the sperm of *M. rosenbergii* display a highly unusual organization. Several questions concerning the function of the sperm structures have been raised. These questions relate to the presence of an acrosome and the role of the spike and radial fibrils in their association with the centrioles. Observations on fertilization are essential in further looking at these questions and are reported in a following paper.

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DIURNAL CHANGES IN THE ACCURACY OF THE HONEYBEE FORAGING RHYTHM

DARRELL MOORE AND MARY ANN RANKIN

Department of Zoology, University of Texas, Austin, TX 78712

ABSTRACT

The ability of honeybees to time foraging visits to an artificial nectar source was analyzed with respect to the time of day of food source presentation. A consistent regimen of orientation and training to the food source, which was available only during a specific one-hour period of the day for each particular experiment, allowed quantitative comparisons to be made among groups of bees trained at different feeding times. Bees trained early in the day showed the most precise time-keeping ability whereas those trained to midday or late afternoon food sources were significantly less accurate. In all of the experiments, the bees anticipated the onset of the training period, but the duration of the anticipatory component of the response was dependent upon the training time. Similarly, other parameters (coefficients of skewness and kurtosis and the shut-down in the number of arrivals immediately after the end of the training time) describing the distribution of foraging flights in time varied according to times in the diurnal cycle when the food source was offered. These results suggest that honeybees more efficiently exploit a daily floral nectar source if it is available early in the morning than if it is offered late in the day. Possible mechanisms underlying the observed differences in temporal orientation are discussed.

INTRODUCTION

The remarkable ability of honeybees to precisely associate the time of day with the presentation of a food reward was first described in the anecdotal reports of von Buttel-Reepen (1900) and Forel (1910) and later in the classical experiments of Beling (1929) and Wahl (1932). Because nectar secretion and pollen presentation of flowers are rhythmic in nature and offered only during specific time intervals of the day, the learned time-sense of honeybees is considered to be highly adaptive (Kleber, 1935). Experiments showing that honeybee foraging rhythms persist under strictly controlled constant conditions of light, temperature, and humidity (Beling, 1929; Wahl, 1932) provide evidence that the bees' ability to remember time is based on an endogenous clock mechanism, and is not driven by external periodic time cues. Unequivocal evidence of the endogenous nature of this biological clock was provided by Renner (1955, 1957) in a series of global translocation experiments. Bees trained to gather food at a fixed time of the day in a closed flight room, under constant illumination and temperature, returned to the food source about 24 hours after the last food reward despite having been displaced over several time zones overnight. However, when the experiments were repeated, not under constant conditions but in the open, the bees re-entrained to the new local time (Renner, 1959). Thus exogenous rhythmic cycles, such as the diurnal repetitions of light and dark, serve to re-set or synchronize the foraging rhythms to the natural environment.

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Later work demonstrated that in the absence of such external time cues (Zeitgebers) honeybee foraging activity occurs with a periodicity of from 23.4 to 23.8 hours in constant illumination (Bennett and Renner, 1963; Beier, 1968; Beier and Lindauer, 1970; Moore, unpublished data) and therefore this rhythm is circadian (period about 24 hours).

The versatility of the honeybee foraging rhythm sets it apart from the activity rhythms of most other animals so far investigated. Honeybees can be trained to visit a food source at any time of the day (Beling, 1929; Wahl, 1932) and, in fact, have been trained to as many as nine discrete feeding times per day (Koltermann, 1974). Bees also learn the locations of widely separate sites and remember to visit them at the correct times (Wahl, 1932; Finke, 1958).

Because foraging bees can associate any time of the daily cycle with a food reward, the honeybee foraging rhythm must involve a "continuously consulted clock" (Pittendrigh, 1958), as do the biological clocks underlying time-compensated celestial orientation in a number of animals, including birds (Hoffmann, 1960; Sauer and Sauer, 1960), bees (von Frisch, 1950), and amphipod crustaceans (Pardi and Grassi, 1955). Despite the long tradition of work in this field, the mechanism of the foraging time-sense remains obscure. One basic question which has not previously been addressed by systematic experimentation is whether the foraging time-sense is equally punctual for all phases of the daylight cycle. That question is the subject of the present study.

MATERIALS AND METHODS

Fifteen field experiments were done, using a consistent training regimen and testing of the time-sense. The procedures used are very similar to the methods employed by von Frisch (review, 1967) for training honeybees to visit an artificial food source. First, worker honeybees were enticed out of the hive with 2 M sucrose solution applied to strips of filter paper which were scented with a drop of floral essential oil and placed at the hive entrance. Next, bees feeding from the sucrose were transported carefully on the filter paper strips to a small feeding table located approximately one meter from the hive entrance. This procedure of transporting the bees was repeated several times. A watchglass (6.25 cm in diameter) containing 2 M sucrose solution and resting upon a 15 cm white filter paper circle was placed in the center of the feeding table. It was kept filled with sucrose solution throughout the training period. One drop of the essential oil, which was used at the hive entrance, was applied via a pasteur pipette to four equidistant spots at the edge ("compass points") of the filter paper disc. After 10–15 bees had flown from the hive to land and feed at the watchglass, the feeding table was moved several meters farther from the hive. This procedure was repeated, allowing about five minutes of feeding at each location. The table was moved in gradually increasing steps until it was located 100 meters from the hive; the entire procedure was accomplished within one hour. During this time, more bees were constantly being recruited from the hive such that at the end of the hour as many as 100 bees or more were typically foraging at the watchglass. Toward the end of this one-hour "orientation" training, the sucrose solution was not replenished but allowed to dry up coincident with the termination of the training period. At this time, the scented filter paper disc was removed and the feeding table and the watchglass were thoroughly rinsed with water to remove all traces of sucrose. The table and watchglass were then quickly dried and the watchglass was re-positioned over a fresh unscented filter paper disc on the table. The feeding table was kept at this location for the remainder of the experiment.

During the three days following orientation, bees were trained at the same time of day as the orientation. Within the one-hour training period, the watchglass was kept supplied with sucrose and the filter paper was scented, as before. Again, the sucrose and scent were promptly removed at the termination of the one-hour training period, but the empty watchglass and unscented filter paper remained on the training table at all times other than the training period.

On the fifth day, designated the "test" day, the watchglass remained empty and the filter paper unscented to eliminate the possibility of any recruitment of bees from the hive due to the presence of sugar or odor clinging to returning foragers. The times of arrivals of bees at the watchglass were monitored from sunrise to sunset. Flights within about 15 cm of the watchglass as well as direct landings on it were scored as arrivals. Individual bees were not marked in these experiments, but because it took a minimum of about three minutes for a bee to make a round trip between hive and feeding station, it was fairly easy to keep track of individual bees once they arrived at the station. Although each bee may make many return visits (Beling, 1929; Wahl, 1932; Moore, Siegfried, Wilson, and Rankin, in preparation), they were not counted more than once per visit.

The experiments were designed to mimic the manner in which a foraging group naturally exploits a newly-found food source. The numbers of bees arriving at the feeding station were not restricted, and foragers were free to recruit their hive mates. On test days, the distribution of arrivals at the training station represented how the bees, as a group, incorporated the training time into their time-sense.

In one additional experiment, the training time was increased to three hours but otherwise conditions were identical to those outlined above. Lilac scent was used for orientation and training in this particular experiment.

Honeybees obtained locally from area beekeepers were used in all of the experiments. Although the bees were not of a pure strain, they were predominantly *Apis mellifera ligustica.* The experiments took place either at Balcones Research Center or Brackenridge Field Laboratory of the University of Texas, which are located within the city limits of Austin, Texas. All of the experiments were done on bright, clear to partly-cloudy, warm days.

The results are displayed on graphs showing the number of arrivals per time, in five-minute intervals, beginning with official sunrise or the first arrival at the feeding station (sometimes a forager would arrive just before official sunrise). Data from the individual experiments were combined into appropriate groups according to the time of day of training and the resulting frequency distributions were subdivided quantitatively into "accurate" and "inaccurate" arrivals. The mean arrival time and the coefficients of skewness and kurtosis were tested in each case by employing the *t*-distribution (Sokal and Rohlf, 1969). Comparisons for significant differences among the groups were made using chi-square analysis. Additionally, differences with respect to skewness and kurtosis from the combined data were compared using the *t*-test.

RESULTS

The foraging experiments were partitioned into three groups according to the time of day in which the training time took place. Training occurred between one and three hours after sunrise in the morning group (Fig. 1). The noon group was trained between five and seven hours after sunrise (Fig. 2), while the evening group was trained between eight and ten hours after sunrise (Fig. 3).

All groups tested anticipated the training time. On test days we observed a

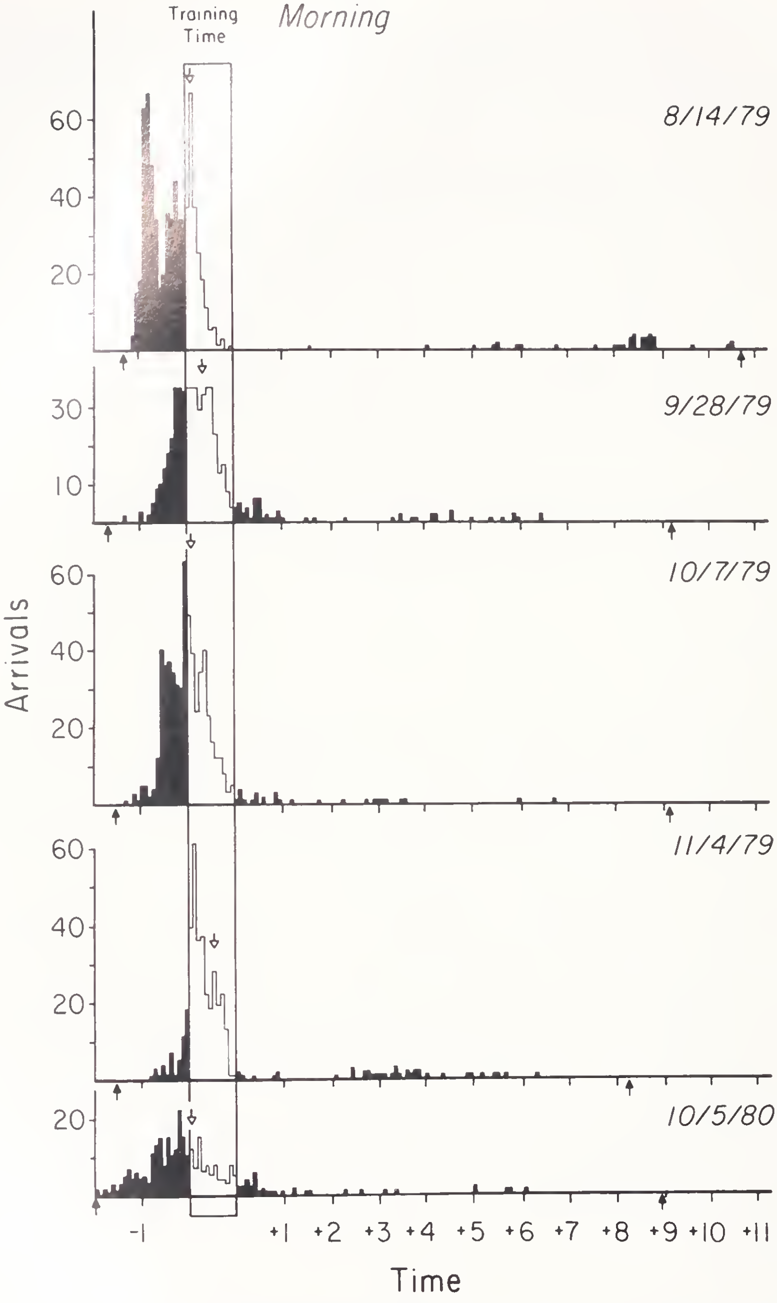

FIGURE 1. Morning group experiments. The distributions of arrivals on test days, at the feeding dish, are plotted in five-minute intervals with sunrise and sunset indicated by upward-pointing arrows. The number of hours preceeding and following the training time (negative and positive values, respectively) are shown at the bottom of the figure. Framed within the vertically-extended rectangle are the one-hour training periods. The mean of each forager arrival distribution is indicated by a downward-pointing arrow above the response curve.

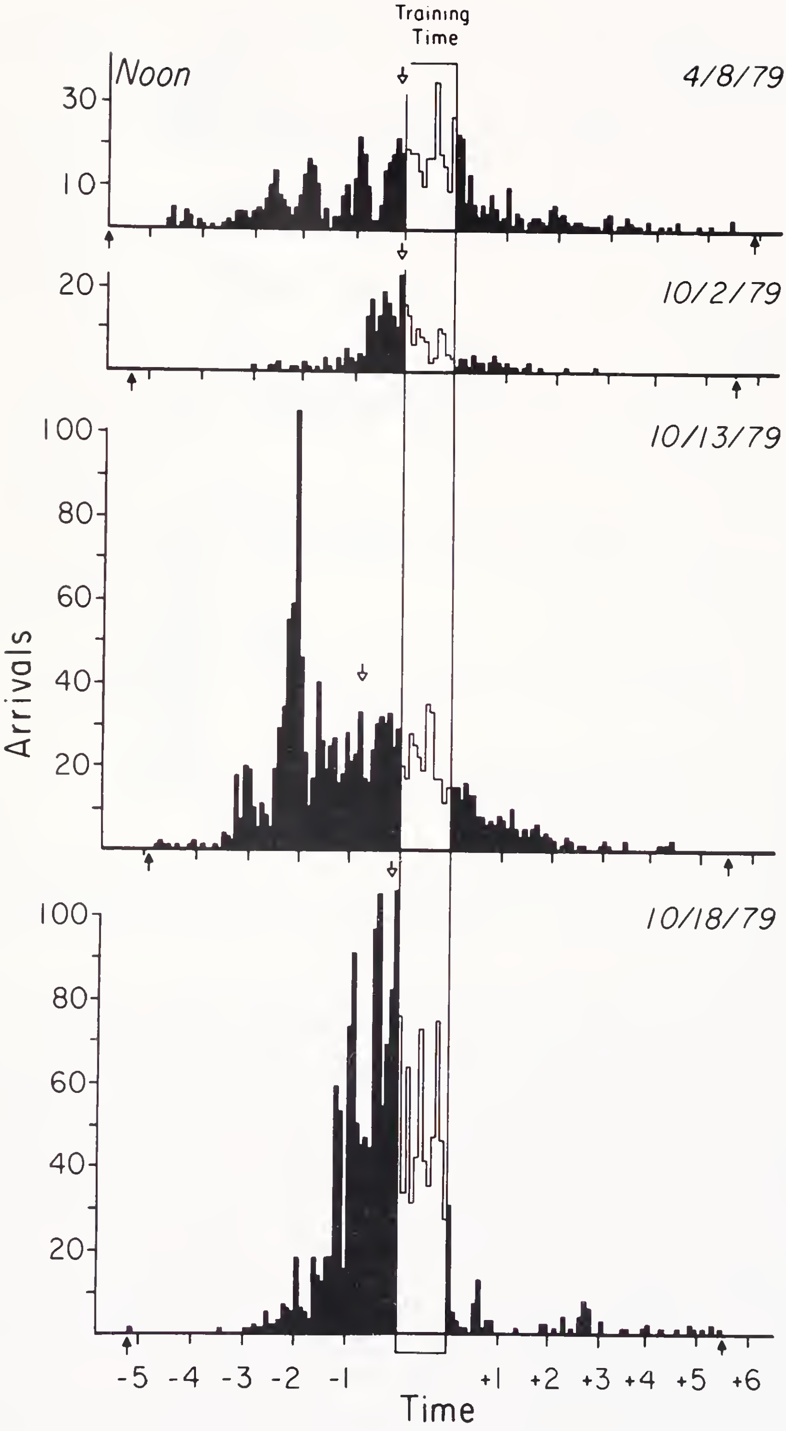

FIGURE 2. Noon group experiments. Details as in Figure 1.

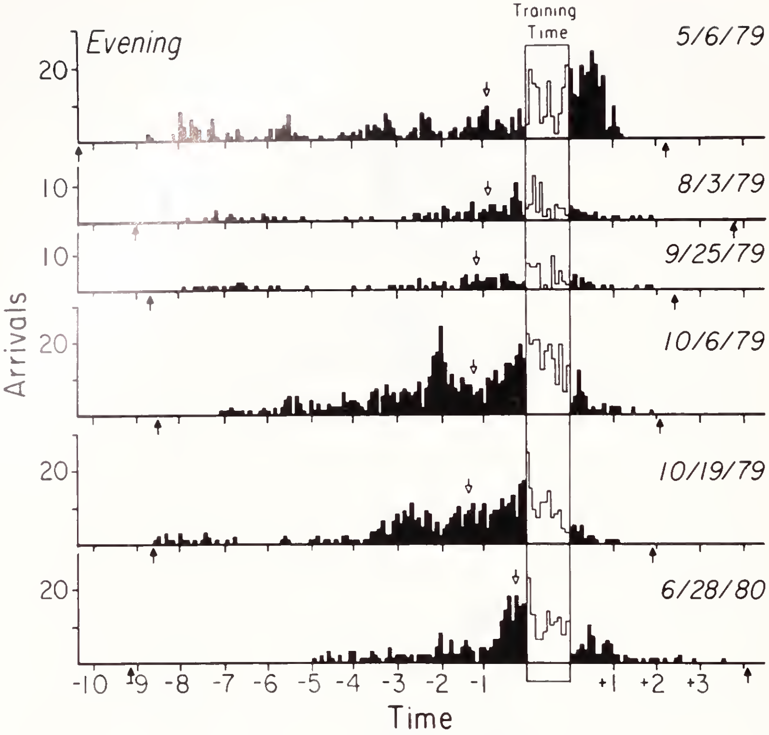

FIGURE 3. Evening group experiments. Details as in Figure 1.

considerable number of arrivals at the foraging station before the training time. This is consistent with the results reported previously (Beling, 1929; Wahl, 1932) and, according to von Frisch (1967), gives honeybees a competitive advantage over other nectar foragers and should not necessarily be construed as an inaccuracy in the foraging time-sense.

On test days, almost all experiments showed less bee activity at the end than at the beginning of the training period. This is to be expected if bees cue on the onset of the training period or, alternatively, if after several unrewarded flights to the food source at the "correct" time, the tendency to make any further return visits is lessened. The importance of the beginning of the training time was demonstrated by a morning experiment in which the training period was extended from one hour to three (Fig. 4). In this experiment, most of the activity (66.2%) occurred in the one hour immediately before and the first hour within the training period. By the end of the training period, the response level was reduced to only 6% of the value at the beginning.

Consistent with the anticipatory nature of the foraging response pattern, most experiments are characterized by fewer arrivals after the training period than before it. The three experimental groups differ markedly, however, in other parameters which describe the temporal distribution of their responses.

In terms of accuracy, or the precision of the foraging time-sense with respect to training time, it appears that the morning group was superior to both the noon and evening groups. To estimate forager accuracy, the total number of arrivals within the training time and within the one-hour period immediately prior to it are des-

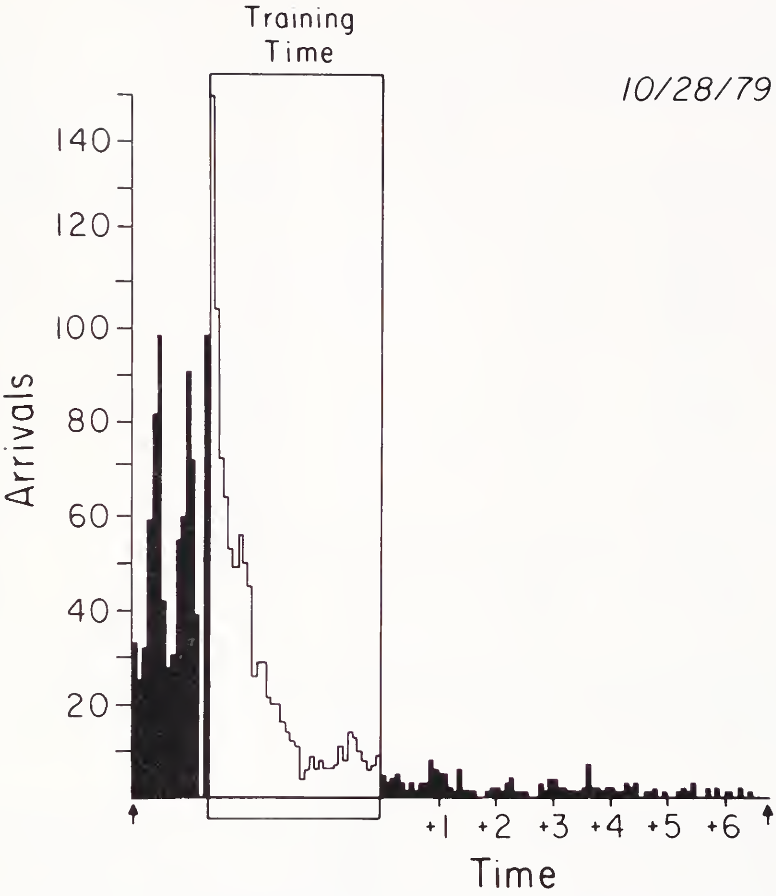

FIGURE 4. Morning group experiment with training duration of three hours. All other details as in Figure 1.

ignated as "accurate" and all arrivals at other times are considered "inaccurate." This measure of time-sense precision assumes with von Frisch (1967) that anticipatory visits are an integral part of the foraging behavior of honeybees and should not be regarded as inaccurate. The average percentage of "accurate" arrivals was 88.0% for the morning, 61.1% for the noon, and 48.9% for the evening experiments (Note that Table I shows percentages for individual experiments). A chi-square analysis demonstrates that the morning group significantly outperformed both the noon and evening groups ($P < 0.001$ in both cases). Also, the noon group was significantly more "accurate" than the evening group ($P < 0.001$).

Another parameter which is important in terms of foraging efficiency is the ability to predict when a nectar source will no longer be available, *i.e.*, to shut down foraging activity at the appropriate time. Only 3.4% of the total number of morning arrivals occurred within the one-hour period immediately following the training time. By comparison, the noon and evening groups exhibited much higher levels of activity at the foraging station after the termination of the training time than did the morning group; 7.3% and 13.2% respectively, of the total responses lie within this time-frame. This result is surprising, especially so for the evening group, since

TABLE I

Data summary for the individual field experiments

Group	Experiment	Hive	Scent	Total number of arrivals	% Accurate	Skewness coefficient	Kurtosis coefficient	No. of 5-min intervals between mean & training time onset**
MORNING	8/14/79	A	Lavender	715	92.2	3.97	17.96	−2
	9/28/79	A	Lilac	551	88.4	3.64	18.32	−5
	10/07/79	B	Lilac	597	94.5	4.37	32.76	−1
	11/04/79	C	Lilac	388	89.9	3.00	11.69	−7
	10/05/80	E	Lilac	295	74.9	2.68	13.91	0
NOON	4/08/79	D	Lavender	770	49.0	0.25*	3.66	+1
	10/02/79	B	Jasmine	295	79.0	0.45*	4.82	+1
	10/13/79	C	Jasmine	1497	38.1	0.58	3.24	+9
	10/18/79	A	Jasmine	1855	78.3	1.67	10.21	+2
EVENING	5/06/79	D	Lavender	549	32.6	−1.22	3.31	+11
	8/03/79	A	None	166	54.2	−1.48	4.62	+11
	9/25/79	C	Lavender	143	50.3	−1.31	3.87	+14
	10/06/79	A	Lavender	706	47.0	−0.78	3.17	+15
	10/19/79	B	Lavender	509	48.5	−1.49	5.58	+16
	6/28/80	E	Jasmine	432	60.9	−0.62	3.89	+3

* All of the coefficients of skewness and kurtosis are statistically significant at $P < 0.001$ except those denoted by a single asterisk, which are significant at $P < 0.01$.

** Negative values indicate that mean occurs before training time onset; positive values indicate that mean occurs after training time onset.

compared to morning foragers, the evening bees have fewer foraging hours left in the day. From a chi-square analysis, the morning group is significantly more adept ($P < 0.001$) at shutting down its activity than is the noon group which, in turn, has significantly fewer arrivals during this period than does the evening group ($P < 0.001$).

Another measure which may be used to quantify the accuracy of a foraging rhythm is the difference between mean arrival time and the onset of the training time. On the average (Table I), experiments in the morning had mean arrival times only 15 minutes before training time onset. Noon experiments were similar in this regard with an average of 16.25 min. In the evening experiments, however, this difference was almost one full hour. It is interesting to note that the means for the morning experiments all occurred within the training period whereas those for the noon and evening experiments occurred before the training time.

Apart from accuracy, it is evident that the response profiles differ markedly among the three groups. The morning experiments (Fig. 1) have arrival distributions with sharper and higher peaks, whereas the noon (Fig. 2) and evening (Fig. 3) arrival distribution curves have broader peaks. The distribution curves of the groups may be quantified using coefficients of skewness and kurtosis (Table I), which characterize the distribution of the responses about their means (Sokal and Rohlf, 1969). All of the morning and noon experiments are significantly skewed to the left. Additionally, all of the distributions are significantly leptokurtic; that is, there are more arrivals near the mean and at the tails of each distribution than in the intermediate regions. When the results from the experiments within each group are combined and normalized with respect to training time (Fig. 5), it can be seen that the skewness coefficients of the three groups differ significantly from one another (*t*-test, *P*

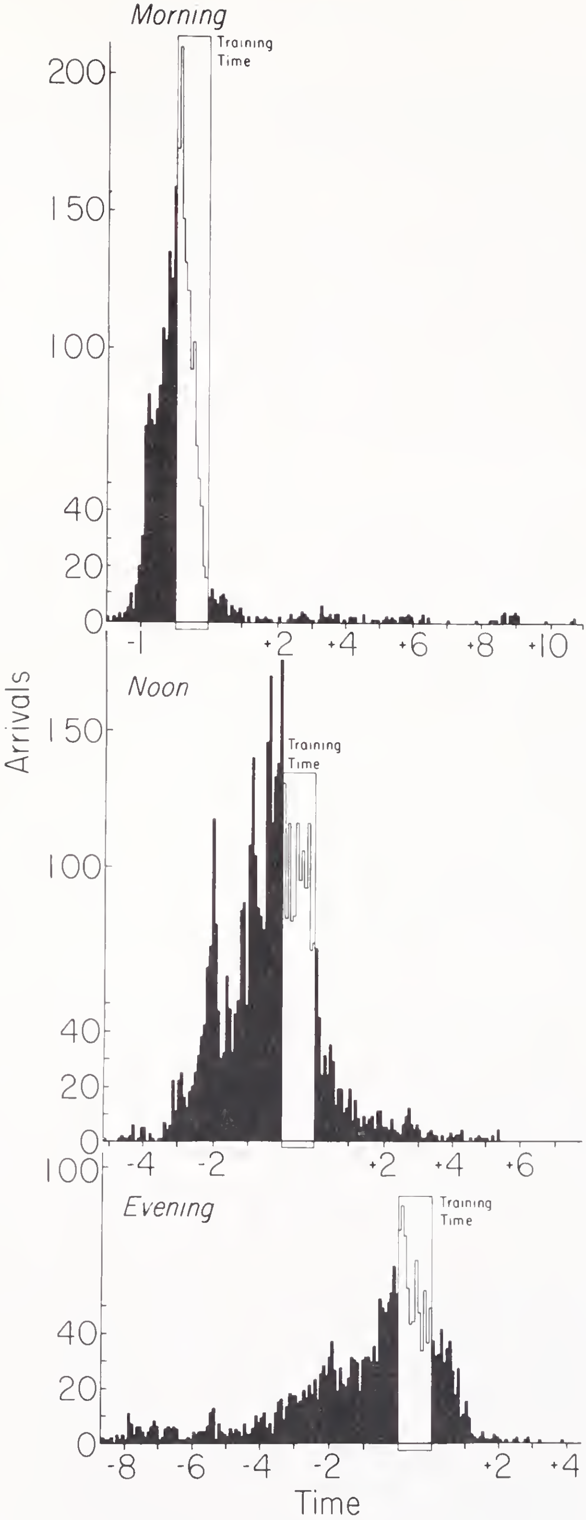

FIGURE 5. Combined data showing the results of all of the experiments within each training group (morning, noon, evening) normalized with respect to training time. Details as for Figures 1–3. Results illustrate the dependence of response profile on the time of day in which training occurs.

< 0.001). It may also be concluded that the morning group was significantly more leptokurtic than both the noon and evening groups (t-test, $P < 0.001$ in both cases) and the noon group was significantly more leptokurtic than the evening group ($P < 0.01$).

Three different scents were used in these experiments and in one experiment no scent was used. As expected, the time-dependent variability of the foraging rhythm was independent of the type of scent used during the orientation and training phases of the experiments (Table I). Comparisons of accuracy measurements, skewness, and kurtosis of individual experiments from the three training time groups (morning, noon, and evening) for the situations in which either the same scent or different scents were used, indicate a consistent pattern; there is a great deal of similarity within the same training group and a great deal of difference between groups.

DISCUSSION

The data reported here indicate that the time-keeping ability exhibited by foraging honeybees is not equal for all times of the day, but rather deteriorates somewhat from morning to evening. Morning training times are remembered with a much greater accuracy than late afternoon sessions, with midday training times intermediate between the two extremes. The observed differences in the accuracy of the honeybee time-sense persist over different times of the year (Table I) suggesting that the phenomenon is largely independent of changing conditions, including variations in natural flora. In experiments with individually marked bees, which will be reported separately (Moore *et al.,* in prep.), we have shown that all of the bees that visited the food dish on the test day of an afternoon experiment also did so on the first two training days (data on individual arrivals were not taken on the third training day). Thus there appears to be no differential recruitment of naive bees in the afternoon as opposed to the morning training groups. Similarly, judging from cuticle coloration, presence of body hairs, and over-all physical appearance, there appeared to be no difference in age distribution in morning as opposed to mid- or late-day foraging groups.

Why there should be a time-dependent variability in the foraging rhythm is not clear. The most energy-efficient strategy for the exploitation of a food source (which itself possesses a diurnal periodicity) by a honeybee colony would be to deploy a large number of foragers such that they arrive at the source just at the onset of nectar secretion (or experimentally, as the training time begins). This would ensure that a maximum number of bees would be able to forage for the entire duration of nectar presentation and, in turn, these bees could recruit a maximum number of new foragers during that time interval. Arrivals too early or too late at the food source must be considered to be energy-inefficient in this scheme. Early arrivals, or anticipation responses, are characteristic of all the experiments described in this study. Morning group experiments, however, show anticipation responses that we consider to be the most efficient, in that they are distributed such that the bulk of early arrivals occur just before the onset of the training time (beginning 15 to 60 minutes before training time). By comparison, midday and evening experiments are characterized by anticipation responses that may begin, at substantial levels, from three to six hours early. In addition, there are significantly fewer late arrivals for morning training times than for midday and evening training times.

There are two distinct possibilities that might account for time-dependent accuracy variation. Accuracy may be affected by signals from the environment which may be used as secondary time cues for the initiation of foraging activity or alternatively, time-sense variation may be entirely endogenous in origin and completely independent of ongoing periodic external time cues. In both cases, the rhythm would be entrained to the natural day-night cycle, with the continuously-consulted program for the time-sense reset each day.

Considering the first possibility, time-dependent accuracy could perhaps be the result of external influences, such as social interactions within the hive (a social Zeitgeber). As the day progresses, there are likely to be frequent interactions occurring within the colony, such as waggle and round dances performed by returning foragers, recruitment of new foragers from the dances, and trophallactic contacts between foragers and hive bees. It might be expected that as food sources make themselves available throughout the day, the probability that a forager would interact with bees from different foraging groups increases. Such encounters presumably could induce a forager to be recruited to a new nectar source or to visit old sources more familiar to it. According to Körner (1939) and von Frisch (1940), foraging groups are spatially separated within the colony, with each group displaying a high degree of flower fidelity. Individual group members do not become active in the hive until the proper time; then they migrate toward the hive entrance and soon thereafter begin their foraging flights. However, there is some suggestion in the work of Medugorac and Lindauer (1967) of a social Zeitgeber influencing the honeybee time-sense. After CO_2-narcosis lasting several hours, bees visited a feeding place at their normal training time and again at a second time, delayed from the first by an amount proportional to the duration of the narcosis. If these narcotized bees were then transferred to another colony, which was trained to an altogether different feeding time, they exhibited three peaks of activity corresponding to the original training time, the narcosis-delayed time, and the host colony foraging time.

The alternative explanation, that the time-dependent variation in the accuracy of the foraging rhythm represents a true circadian phenomenon and thus reflects some inherent property of the endogenous clock(s) controlling this temporal behavior, is intriguing. There are at least three ways in which the endogenous control mechanism might produce the observed variation in precision. First, the clock itself may be less accurate later in the day. Second, the clock may be equally accurate at all times of the day but the coupling between the clock and foraging behavior may loosen as the day progresses. Thirdly, the clock may be accurate at all times and the coupling between foraging behavior and clock may be tight at all times but the behavior may be programmed to change from one part of the day to the next. The first two alternatives imply the existence of some imperfection in the foraging clock system that results in poorer foraging time accuracy later in the day. The last alternative implies that it may be advantageous to visit mid- and late-day nectar sources with greater time variance.

Whether there exists an adaptive reason for poorer temporal orientation later in the day is a difficult question to address. One might speculate that flower nectar rhythms may be less precise and hence less predictable as the day progresses, but little information is available on this point. Alternatively, perhaps the steady flow of early (anticipatory) visits to midday and evening sources is a programmed response to a greater incidence of competition from other nectar foragers. Indeed, it is perhaps more likely that the increased variance in mid- and late-day foraging behavior is adaptive than that there is a limit in the extent to which the clock system can respond to selection pressure for accuracy over the entire daily cycle.

The observations presented here provide the basis for future experimental analyses of the honeybee time sense. Experiments to discriminate between exogenous and endogenous causes for accuracy variation in foraging are already in progress.

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MECHANICAL PROPERTIES OF PERIVITELLINE FIBERS OF SEA URCHIN EGGS AS STUDIED BY APPLICATION OF CENTRIFUGAL AND ELECTROPHORETIC FORCES

NORIKO OSHIMA

Department of Biology, Faculty of Science, Toho University, Miyama, Funabashi, Chiba 274, Japan

ABSTRACT

Fertilized sea urchin eggs are concentrically located in the perivitelline space. However, after centrifugation or treatment with Ca-free sea water, most eggs came to rest on the bottom of the perivitelline space, and few fibers were detected within the perivitelline space by differential interference microscopy. In normal fertilized eggs with the fibers, the egg shifted to the anode under an electric field and returned to its original position after the field was shut off. In centrifuged eggs without the fibers the recovery to the concentric position was not achieved. Such invariable coincidence of the perivitelline fibers and the concentric position of the eggs shows that the fibers are the structure supporting the egg in the central portion of the perivitelline space.

The mechanical properties of these fibers were studied by application of centrifugal and electrophoretic forces. The results indicate that: 1. the fibers show an internal viscous resistance against stretching and 2. tension of the fibers is about 0.9×10^{-5} dyne which is large enough to support the fertilized egg in the perivitelline space.

INTRODUCTION

In a horizontal view of fertilized sea urchin eggs, the egg is situated at the center of the perivitelline space. On finding that the density of the egg is higher than that of the perivitelline fluid, Hiramoto (1954) predicted the presence in the perivitelline space of some unknown structure which supports the egg. He also noticed that by centrifugation or treatment with Ca-free sea water the egg settled to the bottom of the perivitelline space, and suggested lability of the structure.

In 1973 Sato *et al.* found, by differential interference microscopy, many thin fibers attaching the egg surface and emanating in all directions towards the fertilization membrane. They also stated that the fibers exist until the morula stage and that they are easily dissolved by treatment with trypsin, pronase, or urea. Thus it appeared that these fibers are the physical entity supporting the egg in position as predicted by Hiramoto.

In this paper, the mechanical properties of the fibers were studied by application of two kinds of external forces, centrifugal and electrophoretic forces. It will be shown that, though seemingly very fine and labile, the fibers are stiff enough to support the weight of the egg in the perivitelline space.

MATERIALS AND METHODS

Fertilized eggs of the sea urchins, *Anthocidaris crassispina* and *Hemicentrotus pulcherrimus,* were used.

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For electrophoresis, the fertilized eggs were fixed in a gap (about 110 μm) between slide glass and cover glass. Both ends were closed by blocks of agar and connected to electrodes through agar bridges. Magnitude of electric current was changed by a direct current power supply (Mitamura Riken). In order to reduce electric conductivity of the medium so as to attain a definite electric field by weaker current, the fertilized eggs were suspended in sucrose-sea water solution (0.75 *M* isotonic sucrose solution/sea water ratio, 5:3; pH 7.9). The migration speed of the egg within the perivitelline space was determined by timing its transit over one (*A. crassispina*) or two (*H. pulcherrimus*) divisions of an ocular micrometer (one division corresponds to 5 μm) with a stopwatch. An 8 mm cinemicrocamera was also used to analyze the movement of the egg under the electric field.

Ca-free artificial sea water used in the present study was composed of 0.462 *M* NaCl, 0.009 *M* KCl, 0.048 *M* $MgCl_2$ and 0.006 *M* $NaHCO_3$.

The perivitelline fibers were observed by Nomarski differential interference microscope with a 40× objective (Olympus).

Centrifugal breakdown of the perivitelline fibers was routinely done 10–15 min after fertilization at 25°C.

RESULTS

Centrifugation of fertilized eggs

When the fertilized eggs of *H. pulcherrimus* were centrifuged at 125 times gravity for 3–7 min, most of the eggs rested upon the bottom of the perivitelline space (Fig. 1). This observation suggests that a structure supporting the egg is broken by low-speed centrifugation. Centrifugation at 125 g for one min, which Hiramoto (1954) used to dislocate the egg, was not effective in the present preparation.

Observation of fibers within perivitelline space

The fibers within the perivitelline space were observed by differential interference microscopy (Fig. 2). As pointed out by Sato *et al.* (1973), they looked thinner than sperm tails (about 0.2 μm in diameter) of the sea urchin. The fibers link the egg to the fertilization membrane, and their arrangement is radiate and random. This organization of protoplasmic fibers persisted even after centrifugation for one min at 125 g. After centrifugation for more than 3 min, most eggs came to the bottom of the perivitelline space, and only a few fibers were detected in the perivitelline space. Therefore, it may be concluded that these fibers are the structure which keeps the egg in the central portion of the perivitelline space.

The fibers which had been broken by centrifugation were not formed again.

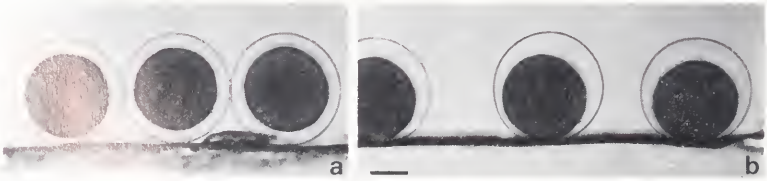

FIGURE 1. Normal fertilized eggs in horizontal view (a) and fertilized eggs centrifuged at 125 g for 3 min (b). *H. pulcherrimus.* Scale bar = 40 μm.

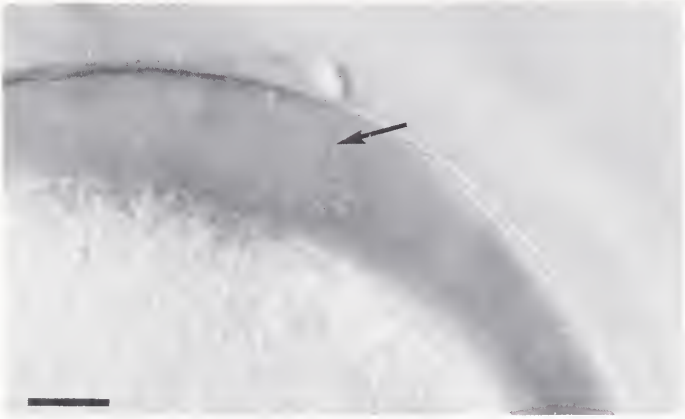

FIGURE 2. Photomicrograph of perivitelline fibers (arrow) observed under differential interference optics. Bar = 10 μm.

Effect of Ca-free sea water

Fertilized eggs were transferred into Ca-free artificial sea water one min after fertilization and washed with Ca-free sea water three times. After this treatment, no fibers formed in the perivitelline space (Fig. 3a). Correspondingly, the egg rested on the bottom of the perivitelline space in horizontal view.

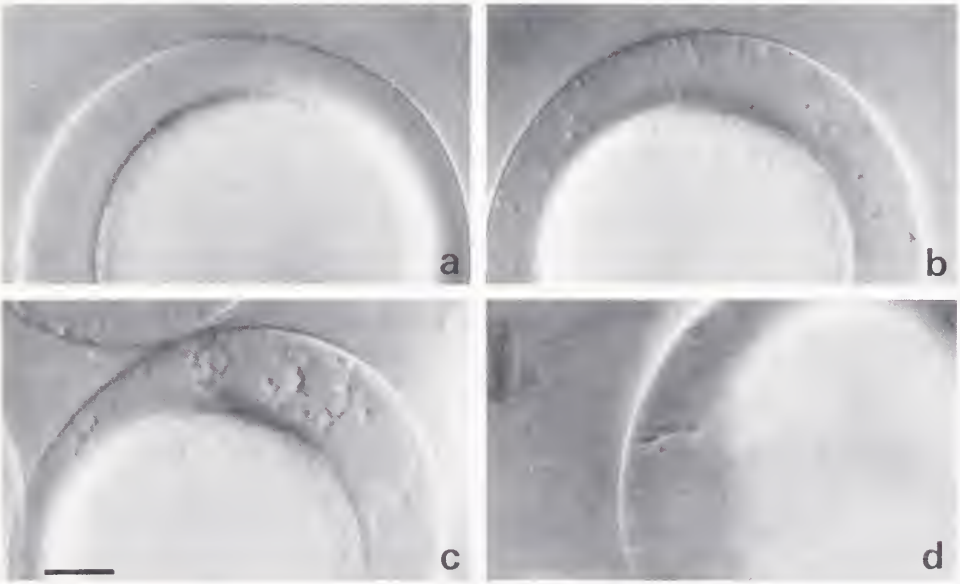

FIGURE 3. a: a fertilized egg transferred to Ca-free sea water one minute after fertilization. b: a fertilized egg transferred to normal sea water from Ca-free sea water (10 min after transfer). The fertilized eggs were immersed in Ca-free sea water for 30 min. c: 20 min after transfer. d: 60 min after transfer. A thick fibrous structure is observed. *H. pulcherrimus*. Bar = 20 μm.

When fertilized eggs kept in Ca-free sea water for 30 min were transferred into normal sea water, many granules soon appeared within the perivitelline space (Fig. 3b). Successively the granules were aggregated and arranged radially (Fig. 3c), which were eventually transformed into the thick fibrous structure (Fig. 3d). This fibrous structure begins to appear some 30 min after the transfer to normal sea water. By this time, half the eggs came to the center of the perivitelline space (Fig. 4). At 90 min after the transfer, most of the eggs were situated at the center of the perivitelline space. Such invariable coincidence of the perivitelline fibers and the concentric position of the egg shows unequivocally that the fibers are the supporting structure.

Electrophoretic movement of eggs within the perivitelline space

When fertilized eggs that are prevented from moving by slight compression with a cover glass are subjected to electrophoresis, the eggs migrate to the anodal side within the perivitelline space (Oshima, 1982). After the egg was shifted to the anode, the electric current was shut off. As shown in Figures 5 and 6, the egg slowly returned to its original position. This suggests that the fibers supporting the egg are not broken down but only stretched by electrophoretic movement of eggs. Observation by differential interference microscopy confirmed this hypothesis (Fig. 7). The fibers at the anodal side appeared to be bent and pressed by the egg against the wall of the fertilization membrane. If the fertilized eggs whose fibers had been broken by centrifugation (125 g, 3 min) were subjected to electrophoresis, eggs once shifted to the anode did not show any movement after the removal current. Therefore, the recovery to the concentric position is achieved by the fibers.

Analyses of electrophoretic movement of eggs within the perivitelline space

Electrophoretic movement of the egg in the perivitelline space is shown in Figure 8. The velocity of the egg near the center of the perivitelline space is fairly constant. In the experiments to follow, the speed determined near the center of the perivitelline space was taken as the velocity of the egg. After centrifugation of the eggs, the speed of the egg became greater than before centrifugation, as was expected (Table I): the lower speed in intact eggs will be due to the existence of the fibers within the perivitelline space. The constancy of speed as seen in Figure 8 suggests that the

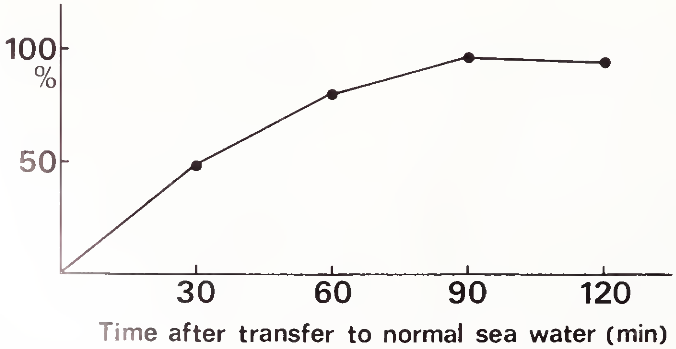

FIGURE 4. Gradual recovery in percentage of eggs situated at the center of the perivitelline space due to transfer to normal sea water after treatment with Ca-free sea water for 30 min. Fifty eggs were used to determine each point. Eggs were obtained from the same animal. *A. crassispina*. 26°C.

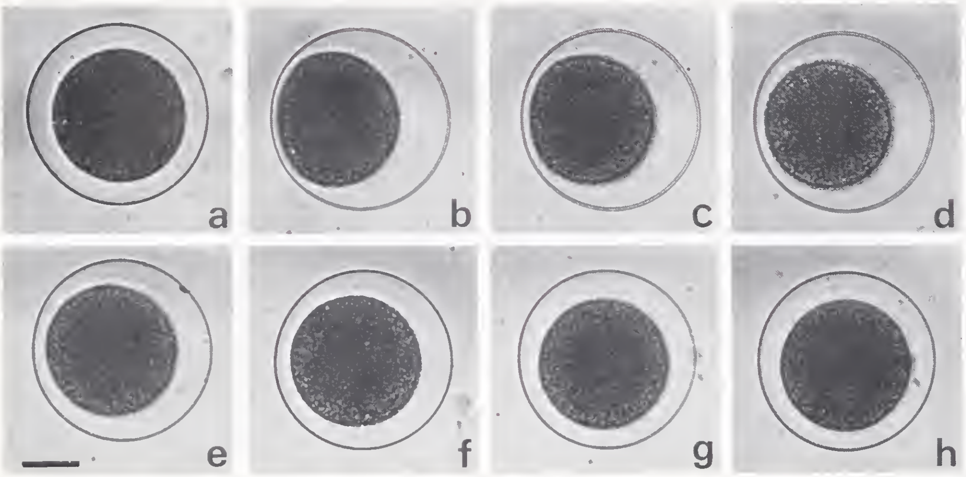

FIGURE 5. Return of egg to its original position after the removal of current. a: control. b: just before the removal of current. c: 25 s after the removal of current. d: 1 min. e: 2.5 min. f: 4 min. g: 7 min. h: 11 min. Potential gradient: 5.5 V/cm. *H. pulcherrimus.* Bar = 40 μm.

resistance to stretching by the perivitelline fibers is due mainly to its viscous properties rather than to its elastic properties, if any.

The relationship between the velocity of the egg and the potential gradient in *A. crassispina* is shown in Figure 9. The velocity of the egg in centrifuged eggs (without fibers) is proportional to the potential gradient. This fact indicates that the perivitelline fluid behaves as a simple Newtonian fluid, which obeys Stokes' law. The constant of the proportionality is about 3.8 (V/cm)/(μm/s).

In the presence of intact fibers in normal eggs, the results are somewhat complicated. The egg did not move with a potential gradient of 0.5 V/cm. Extrapolation

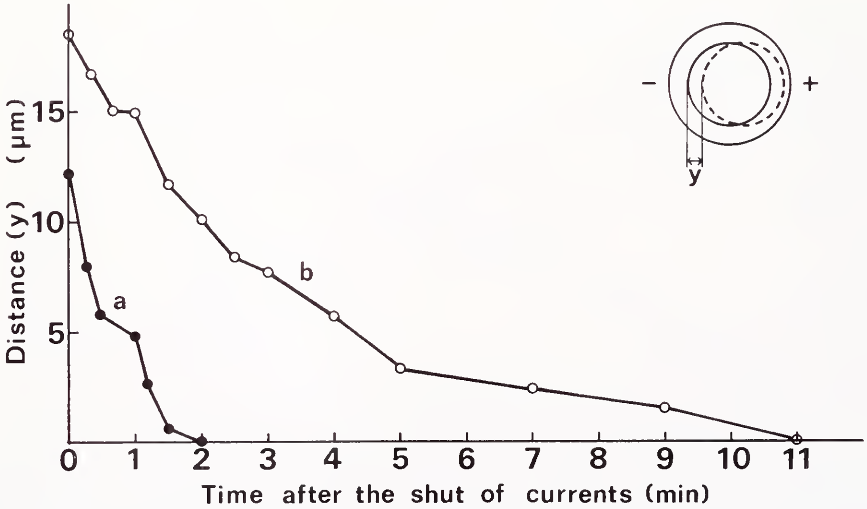

FIGURE 6. Return to the central position of the egg after cessation of electric current. a: *A. crassispina.* 2.7 V/cm for 15 s at 26°C. b: *H. pulcherrimus.* 5.5 V/cm for 15 s at 21°C.

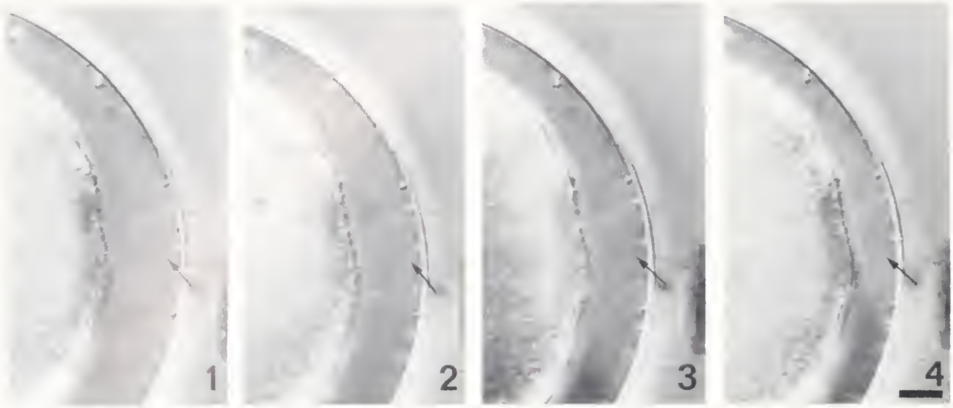

FIGURE 7. Recovery in length of perivitelline fibers (arrows) after the removal of current. *H. pulcherrimus*. Bar = 10 μm.

of the line (Fig. 9) indicates that a threshold for the movement of the egg lies at 0.65 V/cm. Such a 'threshold' indicates the presence of 'resting tension' of the fibers which is counterbalanced by the electrophoretic force of the potential gradient of 0.65 V/cm. This component of resistive force which is independent of stretch may be regarded as due to some surface force or tension at the protoplasmic surface of the fibers.

As seen from Figure 9, the slope of the line of centrifuged eggs (without fibers) is greater than that of normal eggs with intact fibers. This indicates that the fibers show a resistance proportional to the velocity of the eggs (or the speed of stretch of the fibers). This may be regarded as viscous resistance which the fibers show against their stretch. In conclusion, the resistive force due to the fibers is composed of tensional force and internal viscous resistance.

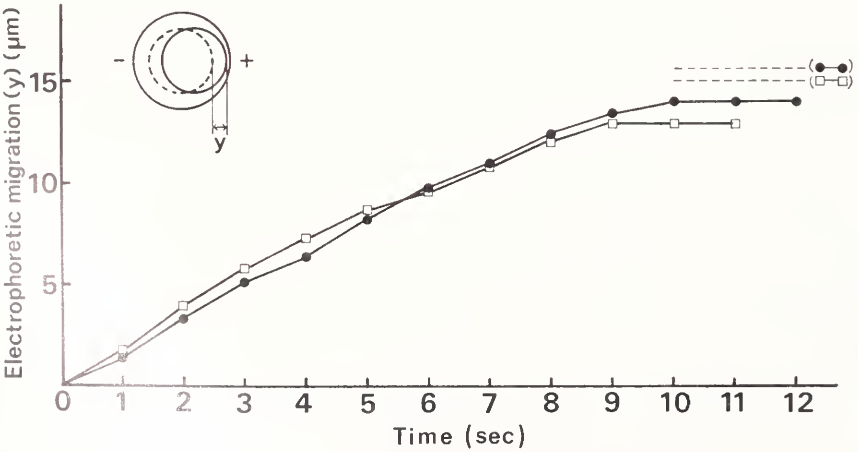

FIGURE 8. Electrophoretic migration of the egg with intact fibers. Two examples are shown. Broken lines are the boundaries of the perivitelline space. Potential gradient: 5.5 V/cm. *H. pulcherrimus*. 22°C.

TABLE I

Migration speed of the egg before and after centrifugation (125 g, 3 min)

Species	Velocity of egg: Before centrifugation	Velocity of egg: After centrifugation	Temperature
A. crassispina	1.06 ± 0.042	1.44 ± 0.067	26°C
H. pulcherrimus	1.47 ± 0.099	2.09 ± 0.143	22°C

Average and standard deviation based on 20 measurements are given in μm/s. Potential gradient: 5.5 V/cm.

Estimation of tension and internal viscous resistance of the fibers

To estimate tensional force and internal viscous resistance of the fibers, the magnitude of external force required for movement of the egg within the perivitelline space was determined by the following procedures.

After centrifugation (125 g, 3 min), the egg velocity falling within the perivitelline space under gravitational force was determined in sucrose-sea water solution following Hiramoto's method (1954). The time required to pass the distance of 5 μm (*A. crassispina*) or 10 μm (*H. pulcherrimus*) at the center of the space was measured 20–50 min after centrifugation and the velocity was determined. The average of ten measurements in *Anthocidaris* eggs and in *Hemicentrotus* eggs are 0.06 and 0.27 μm/s, respectively.

The gravitational force (F) acting on the egg is given as

$$F = \frac{4}{3}\pi a^3(D - d)g, \tag{1}$$

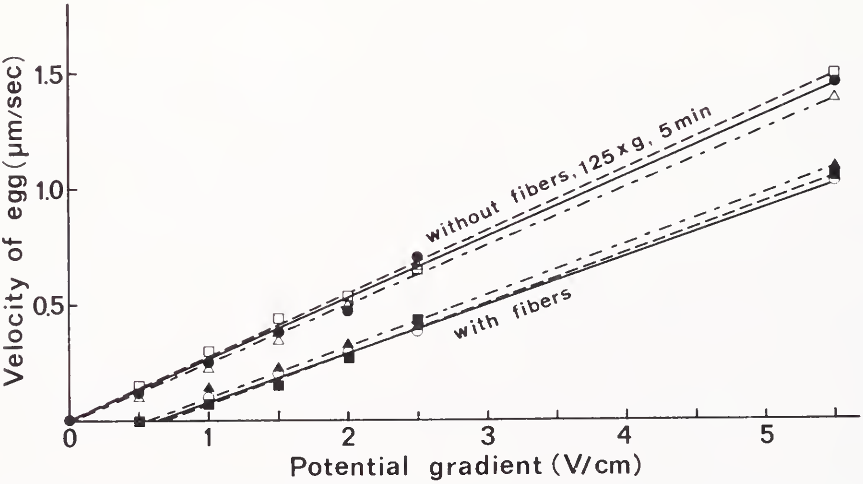

FIGURE 9. Relation between the velocity of egg and the potential gradient. Each point represents the average of five measurements. Results obtained from the same egg are shown by the same symbols. The results shown by the same sort of line were obtained from the eggs of the same animal. *A. crassispina*. 26°C.

where a is the radius of the egg; D is the density of the egg; d, the density of the perivitelline fluid; and g, the acceleration due to gravity. a was about 4.7×10^{-3} cm in *Anthocidaris* eggs, and 4.5×10^{-3} cm in *Hemicentrotus* eggs. The density of the egg (=D) without the fertilization membrane was determined by finding a sucrose-sea water solution isopycnic to the egg. The average value was 1.070 g/cm^3 (*A. crassispina*), and 1.066 g/cm^3 (*H. pulcherrimus*). Similarly, the density of intact fertilized eggs with the fertilization membrane was determined. From these densities and the volume of the egg, the density of the perivitelline fluid (=d) was estimated (*cf.* Hiramoto, 1954) to be 1.066 g/cm^3 (*A. crassispina*), and 1.059 g/cm^3 (*H. pulcherrimus*). Using equation (1), the values of 3.1×10^{-6} dyne (*A. crassispina*) and 5.6×10^{-6} dyne (*H. pulcherrimus*) were obtained for the gravitational force (F) acting on the egg without the fibers, which is counterbalanced by the resistive force due to the perivitelline fluid. From the above results, the external force required for the movement of the egg without the fibers at a rate of 1 μm/s is estimated to be 5.2×10^{-5} dyne in *A. crassispina*, and 2.1×10^{-5} dyne in *H. pulcherrimus*. From this value for *A. crassispina*, together with the proportionality of 3.8 (V/cm)/(μm/s) in Figure 9, the electrophoretic force under unit potential gradient (=1 V/cm) turns out to be 1.4×10^{-5} dyne ($=5.2 \times 10^{-5}/3.8$). The 'threshold' potential gradient, 0.65 V/cm, for the electrophoretic shift of the intact egg with the fibers corresponds to 0.9×10^{-5} dyne, being three times as high as the gravitational force acting on the eggs of *A. crassispina* ($=3 \times 10^{-6}$ dyne). In other words the fibers are stiff enough to support the egg.

Figure 9 was redrawn schematically in Figure 10. In normal fertilized eggs with the fibers, the results mentioned above are summarized by the following expression. External force = A (tension of the fibers) + B (internal viscous resistance of the fibers) + C (viscous resistance due to the perivitelline fluid).

In *Anthocidaris* eggs with the fibers, the above expression is written as external force = 0.9×10^{-5} (dyne) + 1×10^{-5} (dyne) × V + 5.2×10^{-5} (dyne) × V, where V is the migration speed of the egg in μm/s.

DISCUSSION

In intact fertilized eggs with perivitelline fibers, the external electrophoretic force acting on the eggs was shown to be counterbalanced by the total of tensional force, internal viscous resistance of the fibers, and viscous resistance due to the perivitelline fluid. After removal of the current, the eggs that were shifted to anodal side returned to their original positions. Perhaps this is due to tensional force of the fibers at the cathodal side. In this case, tension of the fibers counterbalanced the sum of internal viscous resistance of the fibers against shortening and viscous resistance due to the perivitelline fluid, or tension of the fibers = 1×10^{-5} (dyne) × V + 5.2×10^{-5} (dyne) × V (*A. crassispina*, see above section). As seen in Figure 6, the initial return velocity (taken as the mean velocity during first one min in this study) is about 0.12 μm/s in *Anthocidaris* eggs. Substituting 0.12 (μm/s) to V in the above equation we have 0.7×10^{-5} dyne for the tensional force, which agrees with 0.9×10^{-5} dyne estimated in Results on the basis of the speed of electrophoretic migration of the egg.

To determine the total number of the fibers existing within the perivitelline space, five sheets of microscopical photographs were taken successively at uniform intervals of 2.6 μm near the largest optical section of eggs. From the series of the photographs, the number of fibers existing in the perivitelline space of about 10 μm thick (surface area around this space corresponds to about 10% of the total

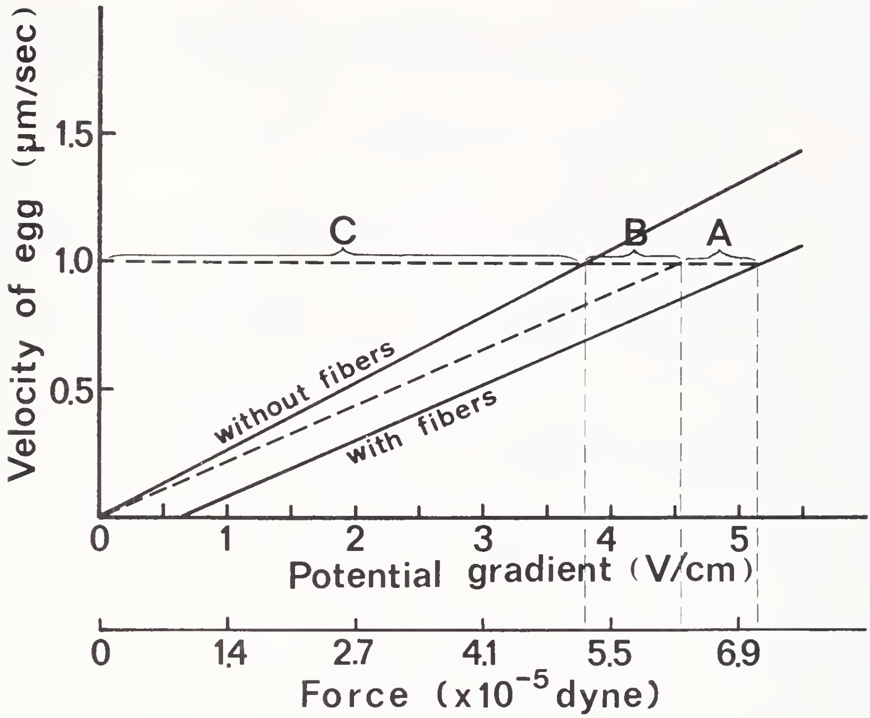

FIGURE 10. Generalized schematic reproduction of Figure 9. A: resistance due to tension of the fibers. B: internal viscous resistance of the fibers. C: viscous resistance due to perivitelline fluid.

surface area of eggs) in which a largest optical section of eggs is located was estimated. In three eggs, the numbers were 108, 148 and 180. Therefore, 1000–1800 fibers will exist in the whole perivitelline space of an *Anthocidaris* egg.

When the electrophoretic force acting on the eggs was below 0.9×10^{-5} dyne, the eggs did not show any movement. If the fibers within the stereoangle of 60 degrees at the cathodal side are effective to support the egg, the number of these fibers is estimated to be 6.7% $(=(2 - \sqrt{3})/4)$ of the total fibers. That is, about 100 fibers will practically support the egg, if the number of the total fibers is 1500. This indicates that a single fiber could withstand the stretching force of 1×10^{-7} dyne. The nature of the structure responsible for such a resistance to stretching is still not clear, but if the resistance is due to the tension working at the protoplasmic surface of the fiber with the diameter perhaps as small as 0.1 μm, then the calculated tension will amount to 2.9×10^{-3} dyne/cm, which approximates to the value of surface tension of a protoplasmic droplet in *Nitella flexilis* (2.3×10^{-3} dyne/cm, *cf.* Kamiya and Kuroda, 1958).

The centrifugal force acting on the egg when it is centrifuged at 125 g was calculated. By using Hiramoto's data (1954) on the density of the egg in *H. pulcherrimus* (1.0715) and that of the perivitelline fluid (1.0369) determined in normal sea water, it came out to be 1.6×10^{-3} dyne. This is forty times greater than the electrophoretic force under the potential gradient of 5.5 V/cm that is the maximum gradient used in this study. By centrifugation at 125 g the fibers will be stretched much more rapidly than by electrophoresis, although their final length is the same as that in electrophoresis, due to a limit set by the presence of the fertilization membrane. Thus, the breakdown of the fibers by centrifugation at 125 g for 3 min will be due to either (1) the rapid stretch or (2) the lapse of time in fully stretched state.

[Appendix]

Viscosity of perivitelline fluid

The resistive force (F) acting on a sphere moving in a fluid is given by Stokes' law, $F = 6\pi a\mu \cdot U$, where a and U are the radius and speed of the sphere, and μ is the viscosity of the fluid.

When the sphere is moving within a definite boundary, the force (F) is written as

$$F = 6\pi a\mu \cdot U \cdot K, \tag{2}$$

where K is a wall correction factor. For the sea urchin egg (with radius A) moving within a spherical perivitelline space with radius B,

$$K = \frac{1 - \lambda^5}{1 - \frac{9}{4}\lambda + \frac{5}{2}\lambda^3 - \frac{9}{4}\lambda^5 + \lambda^6}$$

(3, *cf.* Happel and Brenner, 1965), where $\lambda = A/B$.

The value of $\lambda(=A/B)$ is 0.84 in *Anthocidaris* eggs, and 0.78 in *Hemicentrotus* eggs. Calculation by equations (2) and (3) revealed that the viscosity of the perivitelline fluid in *Anthocidaris* and *Hemicentrotus* eggs are 0.021 and 0.024 poise ($1\ P = 10^{-1}$ Pa·s), respectively. These values, which are about twice as large as that of sea water, suggest the existence of some high-molecular substance in the perivitelline space.

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CHEMICAL ATTRACTION OF NEWLY HATCHED OYSTER DRILLS

DAN RITTSCHOF,[1] LESLIE G. WILLIAMS,[2] BETSY BROWN[3]
AND MELBOURNE R. CARRIKER

University of Delaware, College of Marine Studies, Lewes, Delaware 19958

ABSTRACT

Newly hatched oyster drills, *Urosalpinx cinerea* (Say), that had not eaten prey and had no prior experience in prey detection, were used to screen 25 potential attractants. Of these, odors of intact, living barnacles, *Semibalanus balanoides* and *Balanus eburneus* were most effective, optimally causing upstream migration in over 90% of the snails and retaining detectable activity after 200 fold dilution. The odor of a mixed bryozoan culture evoked a 70% and *Sabellaria vulgaris* a 30% response, whereas responses to *Crassostrea virginica*, oyster valves containing *Polydora websteri*, and *Trypetesa lampas* were low but still significant. Both rheotactic and chemotactic factors were involved in the upstream migration. Behavior reminiscent of trail search was observed in homogeneous dilute stimulus solutions. It is argued that the odor stimuli may be discrete molecules.

INTRODUCTION

The oyster drill *Urosalpinx cinerea* (Say), a predatory muricid gastropod, uses distance chemoreception (for reviews, see Carriker, 1955, 1957; Blake, 1962; Wood, 1968; Pratt, 1974; Ordzie and Garafalo, 1980) to locate a variety of shelled, sessile or virtually sessile prey (Carriker, 1955). Stimulated by an odor, the snail creeps upcurrent, locates the prey, grasps it with its foot, bores through the shell, and consumes the flesh (Carriker, 1955, 1969).

Indiscriminate consumption by *Urosalpinx cinerea* of commercially important species, its extreme hardiness, and *U. cinerea* densities from tens of hundreds per square meter (Carriker, 1955; Hancock, 1959; Wood, 1968) have resulted in numerous drill control attempts. For more than half a century control attempts have been marginally successful (Carriker, 1955). Chemical control by poisons has been unacceptable. There is a clear potential for chemical control based upon the mechanism of attraction to prey.

Results of several studies showed acute distance chemoreception of prey by *Urosalpinx cinerea* (Blake, 1962; Wood, 1968; Pratt, 1974; Ordzie and Garafalo, 1980). Blake (1960) demonstrated that stimulus potency is related to the metabolic activity of the prey. Wood (1968) and Pratt (1974) found that chemotactic responses are modified by diet, a phenomenon described as ingestive conditioning. Efficient testing of adult snails is impeded by their large size, slow speed, state of sexual receptivity and prior feeding experience.

Newly hatched drills have no prior predatory experience, show no cannibalism while in egg capsules, are sexually indifferent (an added bonus, see Wood, 1968) voracious miniature versions of the adult (Carriker, 1957), and should exhibit behaviors unmodified by experience (Carriker, 1957; Thorpe and Jones, 1937). They

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[1] Duke University Marine Laboratory, Pivers Island, Beaufort, NC 28516.

[2] University of Washington, Department of Botany, Seattle, WA 98195.

[3] Battelle Marine Research Laboratory, 397 Washington St., Duxbury, MA 02332

are excellent for low volume bioassays, being about 1 mm in shell height, and more active than adults (Carriker, 1957). Finally, newly hatched drills may be susceptable to biocontrol as they desiccate in a few hours (Carriker, 1957), and will starve to death in a few days at 23°C.

Egg capsules are deposited as long as adult snails remain active, and those deposited in late fall may over winter. One to fifteen newly hatched drills can be obtained from each egg capsule (Hancock, 1956). Thousands of capsules can be readily collected in the field by scraping them from firm substrates to which they are characteristically attached. Development of embryos in capsules can be either retarded by lowering, or accelerated by raising the temperature (Hancock, 1956). A laboratory hatchery of several tens of thousands of egg capsules occupying about two m^3 of space can provide hundreds to thousands of fresh bioassay animals through most of the year.

This paper reports the results of a study designed to assess the potential for chemical control of depredation of the oyster *Crassostrea virginica* (Gmelin) by the oyster drill, *Urosalpinx cinerea.* Our objectives have been to develop a bioassay suitable for biochemical characterization of the molecules responsible for distance attraction of newly hatched snails and to determine potent sources of attractants.

MATERIALS AND METHODS

Egg capsule procurement and treatment

Urosalpinx cinerea egg capsules containing embryos at all stages of development were collected at the inner breakwater in Delaware Bay at weekly intervals between mid-June and October of 1980. Capsules were transported to the laboratory where they were cleaned of debris and stored in 28 g aliquots (~1000 capsules) at 18°C in 15 cm diameter finger bowls containing 200 to 400 ml of filtered (1 μm) sea water. Rates of hatching and development were regulated by temperature (Hancock, 1956). At peak production there were approximately 30,000 egg capsules in the hatchery and 400 to 2,000 snails hatching daily. Newly hatched drills were stored, about 200 at a time, without food in 20 ml plastic cuvettes with plankton screening at both ends to allow exchange of water and metabolites. Cuvettes were stored in finger bowls of filtered sea water.

Stimulus preparation

Odor donor species were collected in local Delaware habitats, when possible, immediately prior to use. Animals not used immediately were stored for 1 to 2 days in the laboratory recirculating sea water system at 20 to 23°C.

Sea water (salinity 31 to 33‰) was trucked at biweekly or shorter intervals from the Indian River Inlet, Delaware, and stored in a 3000 liter holding tank separate from the main laboratory circulating sea water system at 21°C. At intervals, this sea water was pumped through a series of filters, the final one being a 1 μm filter (Filterrite Corp., Timonium, MD). Filtered water was stored in 20 liter and 40 liter glass carboys until used.

Odors were prepared by placing a known wet weight of whole live organisms (1 to 800 g when possible $\simeq$200 g whole wet weight) in a 4 liter aquarium, filling it with 1 μm filtered sea water, and allowing it to stand at room temperature with aeration. Most odors were tested after standing for 2 to 4 h. Subsequently, the aquarium was refilled and the water tested again after standing for 18 to 24 h. In the case of small biomasses (*e.g.*, the barnacle *Trypetesa lampas* and single prey individuals) volumes of sea water were reduced correspondingly.

Biomass of organisms used as stimulus sources varied widely. Serial dilutions of sea water from each potential source were tested to ensure a highly concentrated attractant was not overlooked. Relative strengths of active attractant preparations were determined on a gram live weight basis.

Test apparatus

The assay apparatus consisted of a solution reservoir connected to a flow regulator (Gilmont #2 flowmeter), a stop valve, an assay chamber, and a collection system fitted with a siphon relief. Components were connected by tygon tubing. Assay chambers were modified from a matched set of 2 ml borosilicate glass pipettes. Pipettes were chosen because the 0.1 ml divisions were 10 mm apart facilitating computation of distance traveled by snails, flow discharge, and current velocity. Tips of pipettes were removed at the 1.8 ml mark, mouthpieces were removed 2.5 cm above the 0 ml mark, and a right angle bend was added to the pipette so that the apex of the bend was at the 0.1 ml mark. All freshly formed edges were fire-polished. The 1.8 ml end of each pipette was fitted into a tygon sleeve and could be readily rotated so that the bend in the pipette could be oriented with the open end of the pipette in any position between up and down. In the loading position the open end was up; in the running position the open end was down. During a run the downstream end could be connected to the collection system. The volume of the system, from the stimulus reservoir to the tip of the downstream end of the assay chamber, was 35 ml. In most experiments, four complete assemblies were used simultaneously.

Assay procedure

In the bioassays we used recently hatched *Urosalpinx cinerea* (collected from the capsules within three days) as test animals. All test animals were allowed to equilibrate to ambient temperatures (20–23°C) before an assay. Each test animal was used only once to eliminate any effects of prior exposure to the apparatus or to stimulus water. Preliminary tests were conducted at a variety of flow rates using the endogenous stimulus activity (no added stimulus) of freshly trucked sea water from Indian River Inlet. After several initial experiments with mixed prey stimulus, flow discharge was set to 7.5 ml/min, unless otherwise indicated. Each assay run was initiated by rotating the assay chamber into the load position, filling the chamber with test solution by opening the valve, closing the valve, and loading 10 to 40 test snails into the bore of a test chamber with a camel's hair paintbrush. Flow was restarted at Time 0. At this time the pipette was rotated to a position intermediate between run and load for 45 s. The 45 s period allowed each group of test animals to stabilize in the pipette. Snails landing on their foot attached immediately, and those landing on their shell generally attached within seconds (in agreement with Carriker, 1957). Any snail that crossed the 0.2 ml division of the pipette within 10 min after the start of flow was counted as a positive response. Those snails that attached but did not cross the 0.2 ml division were counted as not responding (Fig. 1). The distance chosen for a positive response effectively separated aroused and creeping snails from those that were just changing position. This distance was often traversed in less than 1 min by aroused snails. At least 35, and usually 50 or more

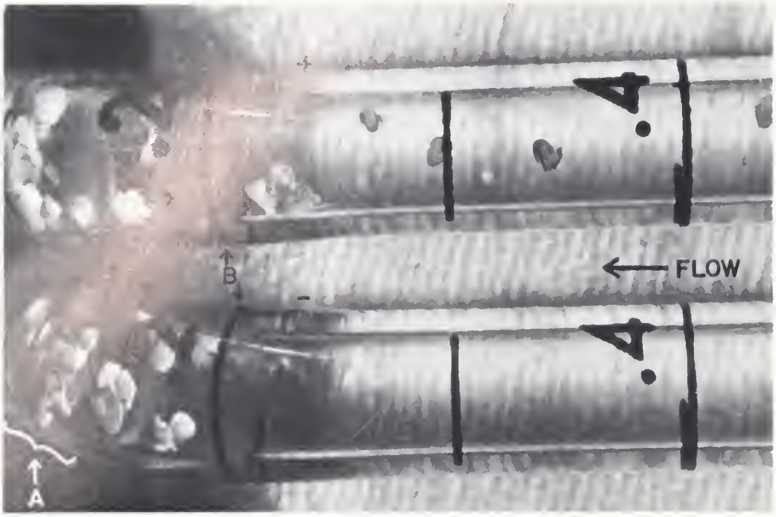

FIGURE 1. Pipette bioassays for chemotactic response of juvenile snails (1–2 mm shell height) to barnacle stimulus. Snails in top pipette migrate upcurrent in sea water bearing barnacle scent, while those in lower pipette remain stationary in a current of filtered, scent-free sea water. Load zone indicated by A and bracket. Positive response criterion 0.2 ml mark (B).

snails were tested in two or more replicate runs for all test solutions. The standard assay procedure was improved by reducing the background stimulus activity of filtered sea water by allowing it to stand several days. Response of snails to background cues was reduced four-fold from 20% to about 5% ($G = 441.7$ $P < 0.005$). This significant reduction in "noise" greatly improved the sensitivity of the assay.

Experiments and observations

Three series of experiments were performed. The first defined bioassay conditions. The effect of current velocities from zero to 360 cm/min was tested on the response of newly hatched snails to sea water with low endogenous attractant activity. Seven current velocities were examined with 100 snails each. The second series screened potential sources of attractant of *Urosalpinx cinerea.* It began with a mixed prey source and then tested individual species from that source. Finally other untested species were screened for attractant production. The mixed prey tests included: oysters (*Crassostrea virginica*), mussels (*Mytilus edulis*), barnacles (*Balanus eburneus* and *Semibalanus balanoides*), hydrozoans (*Eudendrium* sp.), polychaetes (*Polydora websteri*), and miscellaneous amphipods, isopods, and decapod crustaceans. In addition, during storage, sessile organisms had been in contact with the American eel, *Anguilla rostrata,* and the blue crab, *Callinectes sapidus.* Water from this mixed population was compared to a 1 μm filtered sea water control. Next, a series of filter feeding organisms were tested. Included were: hard clams (*Mercenaria mercenaria*); slipper limpets (*Crepidula fornicata* and *C. plana*); bay scallops (*Argopecten irradians*); small barnacles (*Trypetesa lampas*), which burrow into the columella of gastropod shells inhabited by hermit crabs; goose neck barnacles (*Trilasmis inaequilaterale*); and infaunal razor clams (*Tagelus plebeius*). After the series of tests with filter feeding organisms, additional organisms were screened that had not been tested and might release either attractants or molecules that could produce negative chemotaxis. Included were potential predators of oyster drills [lobsters (*Homarus americanus*); starfish (*Asterias forbesi*); and whelks (*Busycon carica*)], conspecifics, potential prey [periwinkles, (*Littorina saxatilis*)], tube worms (*Sabellaria vulgaris*), a mixture of encrusting bryozoans (*Membranipora tenuis* and *Schizoporella irrorata*), and hermit crabs (*Pagurus pollicaris*). The third series used the most potent attractant and examined the effects of stimulus strength and flow on the creeping response of newly hatched snails.

Statistical analysis

Paired contingency comparisons were employed. Responding and nonresponding snails in an experimental group were compared to the same categories in a control group by the G statistic adjusted for unequal sample size and continuity (Sokal and Rohlf, 1969). Controls used to make comparisons were either the response to no added stimulus or to a 1:1000 dilution of a stimulus. The acceptance level chosen was $P < 0.005$. If G values with corresponding probabilities of $0.005 < P < 0.05$ were obtained, experiments were repeated. By these criteria we theoretically made a type I error (*i.e.*, rejecting the hypothesis that frequency of response is independent of stimulus) in about 1 out of 100 tests.

RESULTS

To define the bioassay and to screen potential stimulus sources we performed 248 experiments testing 31,810 newly hatched *Urosalpinx cinerea*. These experiments were designed to investigate parameters a through g as follows:

a. Current velocity

Response of snails to flow increased dramatically from 3% with no-flow to about 20% with a current velocity of 30 cm/min (Fig. 2). There was a more gradual increase in percent response to current velocities from 30 cm/min to 190 cm/min. Maximal response of 33% for any of the current velocities tested was achieved at a velocity of 190 cm/min. The highest current velocity tested, 360 cm/min, resulted in a

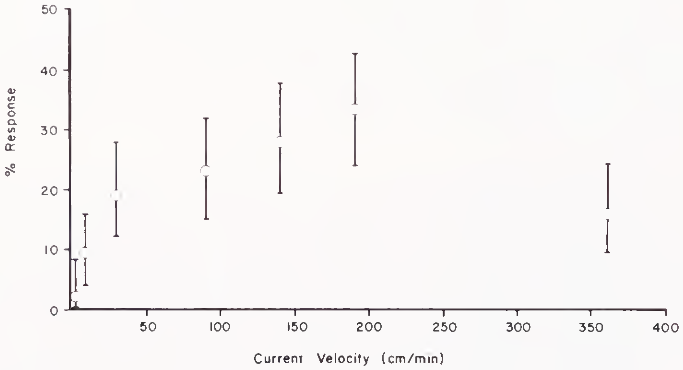

FIGURE 2. Response of newly hatched snails to increasing current velocity with constant background stimulus activity. The assay interval was 10 min and the assay criterion for a positive response was extension of the tip of the siphonal canal beyond the 0.2 ml division of the assay chamber. Test animals that did not move this distance, but that attached were counted as non-responders. Bars represent 95% confidence limits for the samples.

decrease in the rheotactic response to 16%. These data established the flow rate of 10 ml/min to be used in studies of chemotactic response of snails to "mixed prey stimulus."

b. Mixed prey stimulus

The mixed prey stimulus was significantly ($P < 0.005$) active (Fig. 3) at three flow rates (100 cm/min, 86 cm/min, and 75 cm/min) and stimulated about 50% of the newly hatched snails to creep upcurrent.

c. Components of mixed prey stimulus

Experiments then assessed the contribution of each of the major species of mixed prey stimulus to the observed chemotactic response of the snails. The most potent stimulus came from barnacles. In addition to highly significant statistical results, the response of the snails to low dilutions of barnacle stimulus water was greater than 80%, and stimulus water could be diluted 200-fold or more (Fig. 4) and still retain significant (G test $P < 0.005$) activity. Both living *Crassostrea virginica* and empty valves of *C. virginica* containing *Polydora websteri* produced statistically significant stimulus activity. However, response of newly hatched snails never exceeded 21% (Fig. 3). Dilution of oyster stimulus water beyond 10-fold eliminated the response. Water from *P. websteri* in empty oyster valves lost significant activity at any dilution. Snails did not respond to the other 4 species tested.

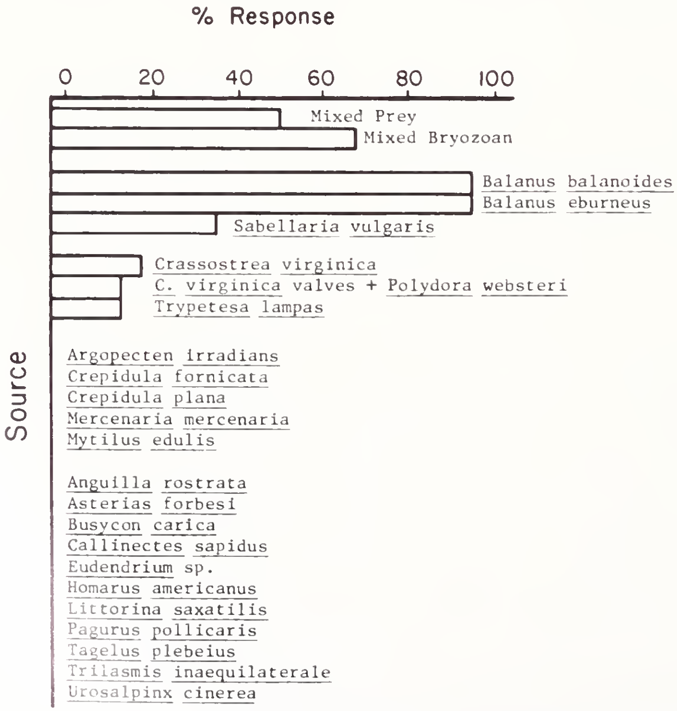

FIGURE 3. Maximal percent response obtained to concentrations of every potential stimulus effluent tested. Responses were grouped into mixed prey, highly stimulatory, significantly stimulatory, nonstimulatory known prey, and nonstimulatory potential prey categories.

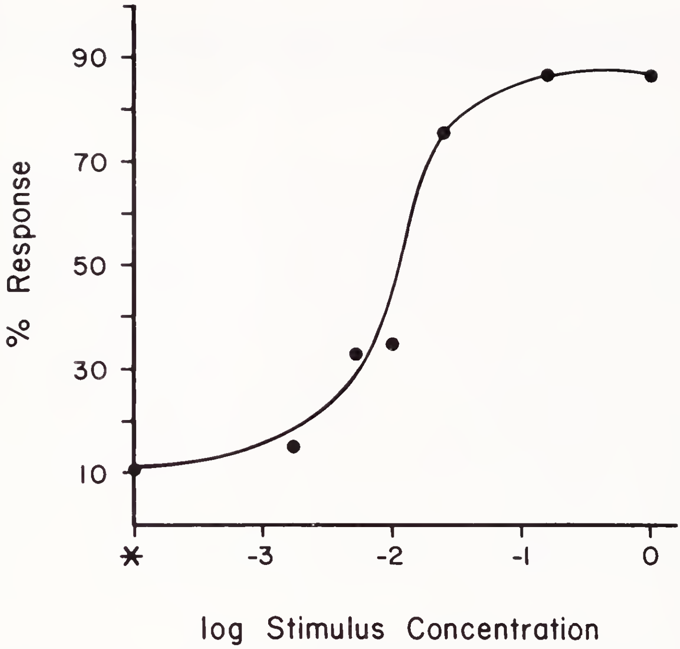

FIGURE 4. The response of newly hatched snails to a dilution series of *Balanus eburneus* stimulus water. Seven grams wet weight of barnacles (11 individuals) were placed in 850 ml of 1 μm filtered sea water and allowed to incubate for two hours. Subsequently the activity response of recently hatched snails was tested over a range of concentrations of stimulus water diluted with 1 μm filtered sea water. Full strength stimulus was considered as having a value of 1. Assay criterion was the same as that described in Figure 2. Flow discharges were 7.5 ml/min. Point represented by * was actually generated by assay of water with background stimulus activity.

d. Individual species stimulus: filter feeders

The first experiments on stimulus water of individual species suggested that not all potential prey of *Urosalpinx cinerea* release attractants that elicit responses from newly hatched snails. Accordingly, we next tested several additional filter feeding organisms. Only water from *Trypetesa lampas* was marginally active, eliciting a low (15%) but significant ($P < 0.005$) response from the drills (Fig. 3). Water from the six additional species did not stimulate the newly hatched predators to creep up-current.

e. Individual species stimulus experiments: other organisms

Of the 8 potential sources tested, only mixed bryozoans and tube worms produced attractants. Waters from mixed bryozoans stimulated up to a 70% response and were active when diluted tenfold. A stimulus associated with tube worms was active only when diluted 10-fold, and evoked a greater than 30% response. No obvious negative responses were made by snails to water from any of the potential predators and no other positive responses were observed.

In summary, screening experiments showed high percentage responses of newly hatched snails to chemoattractants released by intact living barnacles and bryozoans (Fig. 3). In addition, low potency chemoattractants were detected in water associated with tube worms, oysters, empty oyster valves infested with *Polydora websteri,* and the barnacle *Trypetesa lampas*. Of the other 19 organisms tested, only the potpourri of mixed prey stimulus containing the "redolent bouquet" (Pratt, 1974) including stimuli from three species with known attractant-producing abilities was active.

f. Effect of flow

After determining that balanoid barnacles were a potent source of attractant, we tested flow discharge and stimulus concentrations. All rates of flow yielded comparable qualitative information about barnacle attractant concentration (Figure 5a). Although higher flows resulted in higher percent responses at high stimulus concentrations, statistical tests of results of all flows resulted in the same conclusions.

At the three highest flow rates, creep rate increased steadily with each increase in stimulus concentration. Creep rate for the lowest flow showed similar steady increases for intermediate stimulus concentrations with maximum at the 1:10 dilution. Creep rate decreased under conditions of low flow and highest stimulus concentration (Figure 5b). Although percent response did not increase at the lower stimulus concentrations, creep rates of those snails that responded were 25% to 78% higher for the three highest flow rates when rates at no added stimulus and the 1:1000 dilution of stimulus were compared. In general, there was high agreement between creep rate and log concentration for all flow rates.

g. Behavior of newly hatched snails

Newly hatched snails when exposed to homogeneous stimulus water raised the shell and waved the siphonal canal back and forth as described by Carriker (1957). In the presence of an active stimulus some individuals immediately raised their

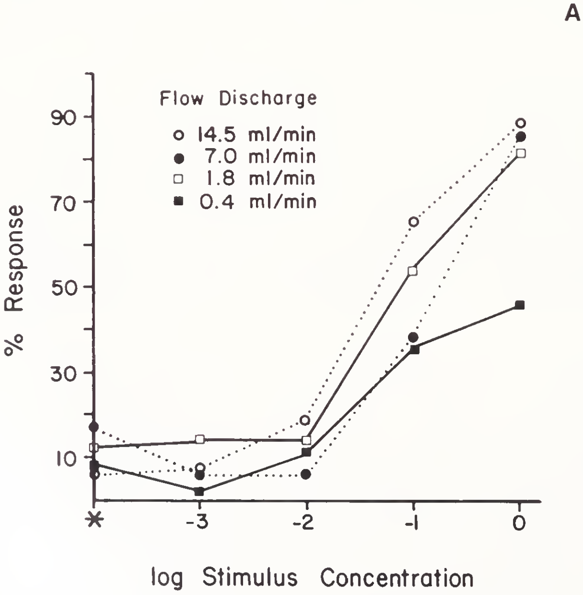

FIGURE 5a. Effect of flow discharge on response of newly hatched snails to dilutions of same stimulus. *Balanus eburneus* stimulus was tested at dilutions and flow discharges shown and by standard assay criteria. Points represented by * were actually generated by assay of water with background stimulus activity.

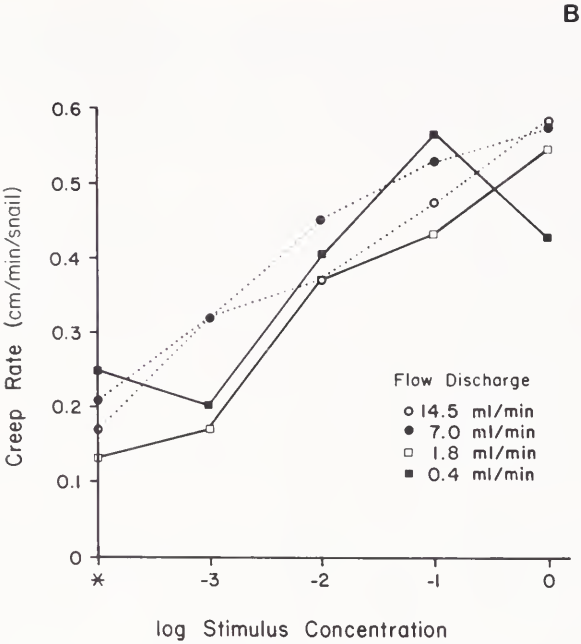

FIGURE 5b. Response of newly hatched snails to various concentrations of *Balanus eburneus* stimulus at different flow discharges. Creep rate, the cm/active snail/min traveled by snails crossing the 0.2 ml division of assay chamber is plotted against log stimulus concentration with undiluted stimulus being taken as 1. * represents assay of water with background stimulus activity.

shells, waved them laterally, and began to creep rapidly, while others remained inactive for varying times and then crept upcurrent. In very dilute stimulus concentrations fewer snails became active, and those that were active did not creep as rapidly as snails exposed to higher concentrations. Exposed to a still weaker stimulus a few snails appeared to be inactive, but, after a 10 min interval, the apparently inactive snails had crept (often in groups but not following each other) up to 1 cm. Some snails crept continuously during the 10 min assay interval over a distance of 16 cm, while others moved for only a small percentage of the time. In the presence of low stimulus concentrations many snails moved upcurrent in a spiral path. In conditions of no stimulus a low percentage crept downstream. In high concentrations, stimulated snails generally crept straight upcurrent.

DISCUSSION

Central in the history of the laboratory study of distance attractants of *Urosalpinx cinerea* has been the choice between water with and without stimulus (Carriker, 1957; Blake, 1962; Wood, 1968; Pratt, 1974; and Ordzie and Garafalo, 1980). In our experiments, rather than requiring that test snails choose between two stimuli, they were scored as to whether or not they moved upcurrent in response to a single homogeneously mixed stimulus (Van Haaften and Verway, 1958; Phillips, 1975; and Lederhendler *et al.*, 1977). Advantages in asking this type of question include a) a homogeneous stimulus source as opposed to a source with unknown mixing characteristics; b) the repeated use of the same test solutions enabling modification and retesting of solutions (important in the isolation and characterization of the active molecular components); and c) the opportunity to dilute solutions in a precisely defined manner. An anticipated disadvantage, a lack of specificity in the response, did not materialize. Savings in time and volume of stimulus waters when compared with previously published snail bioassay procedures are dramatic. The goal that the bioassay be useful for biochemical characterization of attractant molecules has been achieved.

The question of relative potency of stimuli can only be answered rigorously with purified compounds. However, a working understanding of the relative potencies of active waters is important in deciding which stimulus source to purify. We computed relative potencies of the four most potent stimuli, assuming that there is a linear relationship between stimulus strength and response. This is a conservative assumption as it minimizes differences in relative potencies. Computations show a four-fold difference between the relative potency of barnacle and bryozoan stimuli. Barnacle stimulus is over a hundred times more potent than that of tube worms and several thousand-fold more potent than that of oysters.

Odors from at least ten known prey of *Urosalpinx cinerea* and from many other organisms commonly associated with these snails were tested for chemoattractant activity with newly hatched *U. cinerea* (Fig. 3). Only water from bryozoans and *Balanus* spp. stimulated high percentages of snails to creep upcurrent. Curiously, though, adults of newly hatched snails readily consume all known prey species tested. Therefore, as hypothesized for drills by Pratt (1974) and reviewed for fish and crustaceans by Atema (1977, 1980), mechanisms in addition to distance chemoreception must be employed in the feeding response.

The snail predator-prey assemblage has been co-evolving for millions of years (Shrock and Twenhofel, 1953). We hypothesize that virtually every possible mechanism for chemolocation and for disrupting chemolocation has evolved. Testable mechanisms include: 1) chemical camouflaging as described by Fishlyn and Phillips (1980); 2) chemical diversion, *i.e.,* production of a molecule by one prey species, possibly oysters, that facilitates the predatory response to a second prey species, possibly barnacles (a monosodium glutamate effect) similar to that described by Rombauer and Becker (1973) and 3) chemical anosmia; *i.e.,* production of molecules by prey that render the predator unable to smell. It seems unlikely that the latter two mechanisms would evolve unless they were cost-effective (*i.e.,* by-products of other aspects of metabolism).

Our observation that newly hatched snails are attracted from a distance only to barnacles and bryozoans would appear to be in conflict with published work showing that adult *Urosalpinx cinerea* can locate *Argopecten irradians* from a distance (Ordzie and Garafalo, 1980) and some of our own work (Williams and Brown, unpub.) that demonstrates distance chemoattraction of adult *U. cinerea* to oysters. These apparent discrepancies can be readily explained by invoking the phenomenon of "ingestive conditioning" (Wood, 1968). That is, *U. cinerea* can be conditioned in certain circumstances to locate intact individuals of some species that it has previously consumed, given sufficient ingestive experience. This phenomenon is similar to that observed in feeding of tuna (Atema *et al.,* 1980) and lobsters (Derby and Atema, 1981), and in host location of pea crabs (Derby and Atema, 1980) and scale worms (Dimock and Davenport, 1971). Also, one might hypothesize that attractant preferences are manifested in a developmental sequence, similar to that demonstrated for water snakes, *Nerodia erythrogaster* (Mushinsky and Lotz, 1980).

Solutions presented to snails in the bioassay of this study were homogeneous and contained no concentration gradient. Therefore, the creeping response of snails was a product of the integration of stimulus molecule information with rheotactic information (Van Haaften and Verway, 1958; Phillips, 1975). Under ideal conditions creeping tended to be in a straight line. At other combinations of stimulus and flow, response of snails was not as high and their creeping behavior was also altered. At low stimulus concentrations, for example, responding snails often crept in spiral paths suggestive of a search behavior. A search mechanism would be beneficial to snails for location of potent stimulus trails. A second altered response was observed at high stimulus concentration and low flow. Responses in these conditions were lower than responses observed either at higher flow and the same stimulus concentration or at a lower flow and lower stimulus concentration. These observations suggest that in calm water snails may use either secondary prey location mechanisms that are not dependent upon rheotactic information or that they may stop until a current or eddy provides additional rheotactic information about the location of the prey.

Newly hatched snails must travel a relatively long distance (generally several centimeters) from their capsules to their first prey. In addition to the obvious function of prey location, distance chemoreception hypothetically can serve secondary functions such as dispersal and predation avoidance. The newly hatched snail is probably the major dispersal stage of this species (Carriker, 1957). Furthermore, encapsulation of young stages should provide considerable selective pressure for mechanisms facilitating dispersal and minimizing predation. Other mechanisms functioning to maximize dispersal and minimize environmental hazards include positive thigmotaxis, negative geotropism at summer temperatures, positive phototropism to intermediate light levels, speed of movement, and the ability of young snails to raft on debris or on surface films with the aid of a proportionally large foot (Carriker, 1957). Given that the period associated with hatching is critical in the life history, it should be expected that every sense, including those involved in chemoreception, would evolve to direct newly hatched snails to a predator-free, desiccation resistant, well stocked habitat such as that inhabited by bryozoans and barnacles.

The specificity of response of newly hatched drills could be elicited at the molecular level by either a) a mixture of molecules, a chemical picture (Atema, 1977, 1980), or b) a small number (as few as one kind) of specific molecules, analogous to a word (Rittschof, 1980a, b) much like a hormone, relatively high in information content. Although both mechanisms are probably operative in different chemosensory systems and under different circumstances (and in fact may be logical extremes of the same mechanism), it is likely that the latter mechanism is operative here. Turbulence and mixing in the case of a single type of organism would simply dilute a chemical picture without altering its proportions. However, should more than one type of organism (each with its own molecular mixture of characteristic proportions) occur in an area, then turbulence and mixing would result in a mixture that did not resemble the proportions of any of the individuals. On the other hand, other than modification in background to signal ratio, specific chemical words would arrive at receptors as intact units even in turbulent and diverse environments. The latter requires that snails integrate complex flows with stimulus, which they do readily in flow systems resembling waterfalls and pools. Singular molecular forms carrying distinct messages occur universally in life systems as hormones. These are operative as attractants in slime molds (Bonner, 1959) and in distance chemoattraction of white blood cells (Schiffman *et al.,* 1975; Showell *et al.,* 1976) and in distance chemoattraction of hermit crabs to gastropod predation sites (Rittschof, 1980a, b).

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THE DEVELOPMENT OF A SEA ANEMONE TENTACLE SPECIALIZED FOR AGGRESSION: MORPHOGENESIS AND REGRESSION OF THE CATCH TENTACLE OF *HALIPLANELLA LUCIAE* (CNIDARIA, ANTHOZOA)

GLEN M. WATSON AND RICHARD N. MARISCAL

Department of Biological Science, The Florida State University, Tallahassee, FL 32306

ABSTRACT

Three intermediate catch tentacle morphs were observed in the sea anemone *Haliplanella luciae* during catch tentacle development.

Stage 1 catch tentacles, characterized by swollen bulb-like regions along their length, were histologically similar to feeding tentacles.

Stage 2 catch tentacles, which tapered normally along most of their length and then constricted near the tip, were characterized by the presence of feeding tentacle cnidae in the tentacle coelenteron as they were removed from developing catch tentacles. Numerous cnidoblasts appeared in stage 2 tentacles and then synchronously matured into small holotrich nematocysts, a cnida characteristic of mature catch tentacles.

Stage 3 catch tentacles were characterized by the appearance of many large holotrich nematocysts. Such tentacles appeared similar to mature catch tentacles with wide, opaque, blunt tips. However, stage 3 catch tentacles had fewer large holotrichs per total tentacle cross section than mature catch tentacles.

The numbers of large and small holotrich nematocysts decreased in regressing catch tentacles, which tapered to opaque, pointed tips. However, these cnidae did not move to the coelenteron as before but instead migrated to the epithelial surface. This migration suggested that they were externally expelled from the tentacles.

INTRODUCTION

Two types of tentacles occur in certain acontiate sea anemones. One type, the typical feeding tentacle, is a translucent, slender structure that gently tapers from its base to a pointed tip. The second type of anemone tentacle, known as a catch tentacle, is opaque, about twice as wide as a feeding tentacle and blunt-tipped (Williams, 1975). Feeding tentacles usually move in concert in order to capture prey and bring them to the mouth. In contrast, catch tentacles move singly in a so-called "searching" behavior in which they can extend to three or four times their resting length, gently touch the substratum, retract and re-extend. This catch tentacle searching behavior was first described by Gosse in 1860 and later by Carlgren (1929), who also found that catch tentacles have a different cnidom (nematocyst complement) from feeding tentacles. Carlgren suspected that catch tentacles were specialized for feeding. Hence, he named the structures "*fangtentakeln*" (=catch tentacle). Hand (1955) observed that "materials" adhered to catch tentacles of the anemone *Metridium senile* and also noted that catch tentacles regressed into feeding tentacles in starved, isolated animals.

Williams (1975) was the first to recognize that catch tentacles were used in aggressive interactions among anemones. Food items that were touched to catch

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tentacles of the anemone *Haliplanella luciae* did not adhere to the tentacles, nor were the tentacles brought to the mouth. Williams cited unpublished observations by P. R. G. Tranter of the Plymouth Marine Laboratory that catch tentacles were used in intraspecific and interspecific aggressive interactions among the sea anemones *Cereus pedunculatus, Sagartia elegans,* and *Sagartia troglodytes.* Purcell (1977) fully described intraspecific aggressive behavior involving catch tentacles among different color morphs (non-clonemates) of *M. senile.* Following mutual feeding tentacle contact with a non-clonemate individual, a single catch tentacle elongates (as previously described for "searching" behavior). The elongated catch tentacle moves toward the "opponent" and its tip attaches (upon contact) to the upper column or tentacles of the opponent. The catch tentacle breaks slightly behind the tentacle tip as it is withdrawn, thereby leaving the autotomized tentacle tip attached to the "victim." Severe necrosis develops in the victim at the site of the attached catch tentacle tip, occasionally leading to the death of the victim.

In other experiments, Purcell found that catch tentacles developed from preexisting feeding tentacles in *M. senile* when different color morphs (non-clonemates) were crowded into a small aquarium. Since Carlgren (1929) had reported that catch tentacles have a different cnidom from feeding tentacles, this meant that feeding tentacle cnidae must somehow be replaced by catch tentacle cnidae during catch tentacle development. Spirocysts are the dominant cnida in feeding tentacles while holotrich nematocysts are the dominant cnida in catch tentacles (Hand, 1955; Williams, 1975; Purcell, 1977). Purcell (1977) demonstrated that such a turnover takes place during catch tentacle development by counting cnidae from squashes of developing catch tentacles using light microscopy. However, the morphogenetic processes that accompany catch tentacle development remain poorly understood.

The present study describes for the first time the morphogenetic processes involved in catch tentacle development and catch tentacle regression in the sea anemone *Haliplanella luciae.*

MATERIALS AND METHODS

Animal collection and maintenance

Two different clones of the sea anemone *Haliplanella luciae* were removed from rocks or small oyster clumps near the Florida State University Marine Laboratory, Turkey Point, Florida. A third *H. luciae* clone was collected by L. L. Minasian at the mouth of the Indian River in Delaware. Monoclonal *H. luciae* stock cultures were established following frequent fission events, beginning with isolated individuals of each *H. luciae* clone. Stock culture anemones were kept in culture dishes filled with natural sea water (28–30‰), which was changed daily and held at 17–19°C. The sea anemones were fed to repletion twice weekly with freshly hatched *Artemia* nauplii according to the methods of Minasian and Mariscal (1979).

Specimens of the sea anemone *Diadumene gracillima* (which can have catch tentacles) were collected from oyster clumps near the FSU Marine Lab. Unlike *H. luciae, D. gracillima* seldom reproduces asexually. Thus, experimental *D. gracillima* were not monoclonal. *Diadumene gracillima* was maintained in culture dishes filled with sea water by the methods described above for *H. luciae* stock cultures.

Induced catch tentacle development

Three different biclonal, intraspecific cultures were established using the three *H. luciae* clones. Forty-eight organisms, twenty-four of each clone, were placed in

70 mm diameter culture dishes filled with sea water to a height of 10 mm (40 ml) in order to crowd the anemones and insure frequent tentacle contacts throughout the culture. In addition, a single interspecific anemone culture was established using twenty-four monoclonal *H. luciae* mixed with an equal number of *D. gracillima* in a culture dish filled with 40 ml of sea water. In order to control against possible effects of crowding on catch tentacle development, a control culture of forty-eight monoclonal *H. luciae* was established in a culture dish as before.

The procedures described above were intended to stimulate the development of catch tentacles in *H. luciae*. Anemone cultures were screened at approximately one-week intervals for developing catch tentacles, mature catch tentacles, and regressing catch tentacles.

Histology

Specimens were anesthetized in their culture dishes using a one-to-one solution of 7.5% MgCl and sea water. All tentacles were fully relaxed in length. However, catch tentacle developmental stages characterized by constrictions in diameter (as described below) held their typical shape in the anaesthetic and throughout subsequent tissue processing. Developing catch tentacles and regressing catch tentacles were removed from the experimental animals at the interface of the tentacle base and oral disc using fine forceps, drawn into disposable pipettes and dropped into 100% formalin. For small pieces of tissue (*e.g.* anemone tentacles), it was discovered that 100% formalin gave superior results to other fixatives. Mature catch tentacles were removed from freshly-collected animals or from anemones held in long-standing laboratory cultures, then fixed using the technique described above. Following a four-hour fixation, the tissue was washed twice in distilled water, dehydrated in a graded ethanol series, cleared with toluene, and embedded in Paraplast Plus. Ten-micrometer cross sections were mounted and stained in azure a, eosin b for forty minutes after Lillie (1965), then viewed through a Nikon compound microscope using Nikon 15× oculars and a Nikon 100× plan, oil immersion objective lens. The three distal-most tentacle sections having gastrodermal tissue (to avoid counting tentacle tip shavings) were observed and their cnidae were grouped into three categories as follows.

1. Mature cnidae. All "mature" nematocysts (deep blue in azure a, eosin b) were identified by capsule morphology after Mariscal (1974). Mature spirocysts stained dark red in azure a, eosin b and were conspicuous by their color.
2. Cnidoblasts. Since this term is often used incorrectly, it should be pointed out that we use the term "cnidoblast" to refer to the developmental stages of cnidae only and not to the mature structures. Cnidoblasts stained pink in azure a, eosin b.
3. Gastrodermal cnidae. These are mature cnidae that are located in the gastrodermis or free in the coelenteron at the tentacle tip.

The average number of cnidae of the above categories was calculated from three sections per tentacle and compared for each of the following tentacle types: feeding tentacles, developing catch tentacles, mature catch tentacles, and regressing catch tentacles.

Initial examinations showed that the tentacle section cnidom (nematocyst complement) could differ among the tentacle types in (1) the total number of cnidae per section (*i.e.*, the "cnidom size") and/or in (2) the percentages of various cnida types in relation to the tentacle section cnidom. Therefore, these two parameters were studied in detail and are described below.

Cnidom size

The cnidom size is a measure of the number of cnidae per tentacle tip cross section. In this study, the feeding tentacle cross section cnidom (of 114.7 cnidae, n = 6) was used as a standard "unit cnidom." The cnidom size for a given tentacle type was calculated by dividing the average number of cnidae per tentacle cross section by the average number of cnidae (114.7) per feeding tentacle cross section. Thus, tentacle types with fewer than 114.7 cnidae per tentacle section had a corresponding cnidom size that was smaller than 1.00 unit cnidom, while tentacle types with more than 114.7 cnidae per section had a somewhat larger cnidom size than 1.00 unit cnidom.

Catch tentacle morphogenesis and regression involve substantial tentacle growth and degrowth (*i.e.*, a decrease in tentacle diameter) and thus result in significant changes in tentacle shape. Such changes occur in addition to changes in the tentacle cnidom. Methods of counting cnidae per unit circumference or area are not suited for use in this study since tentacle circumference (or area) can change along with the tentacle cnidom and thereby mask changes in the cnidom. The method of counting all of the cnidae per cross section for each tentacle type avoids such complications caused by tentacle growth (or degrowth) processes.

Cnida percentage

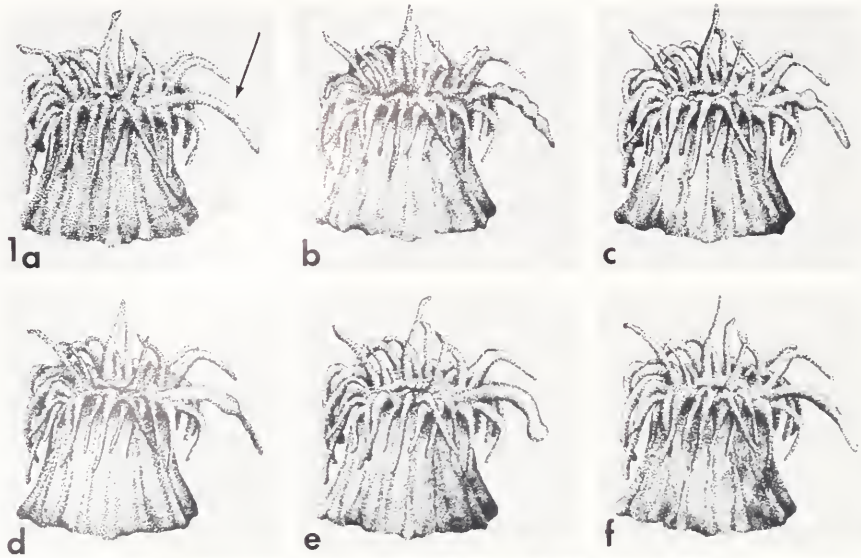
So that changes in the tentacle cnidom that might occur without affecting cnidom size (*e.g.*, a balanced addition and deletion of different cnida types) could be detected, the percentage of each cnida type was determined in relation to the total cross section cnidom for each type of tentacle. For example, as described below, feeding tentacles had an average of 114.7 total cnidae per tentacle section. Of these 114.7 cnidae, 65.8 (or 57.3%) were spirocysts.

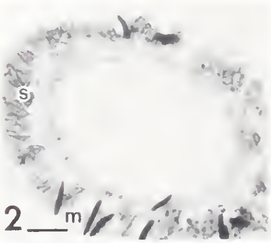
RESULTS

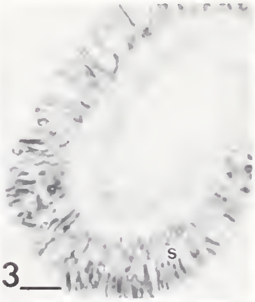
Three distinct developing catch tentacle morphs were observed in *H. luciae* held in intraspecific and interspecific anemone cultures. These were labeled "stage 1," "stage 2," and "stage 3," according to their order of appearance in anemones during catch tentacle development. There was no appreciable histological difference between developing catch tentacles of *H. luciae* held in interspecific cultures and those of *H. luciae* held in intraspecific cultures, although the former developed more rapidly (Watson and Mariscal, in prep.).

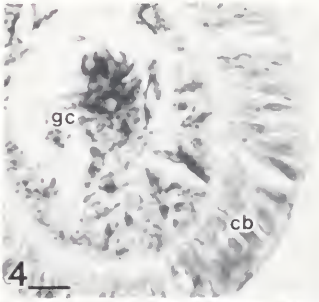
Tentacle morphs

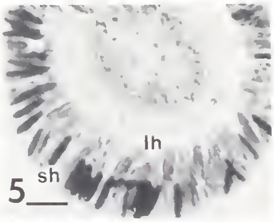
A feeding tentacle gently tapers from its base to a pointed tip (Fig. 1a). On the other hand, the stage 1 intermediate catch tentacle has temporary bulb-like regions (one to several) that stand out along its length for a few hours at a time. The multiple-bulb morph is shown in Figure 1b and the single-bulb morph in Figure 1c. The stage 2 intermediate catch tentacle tapers normally along its length and then constricts sharply near the tip for a few hours at a time (Fig. 1d). The stage 3 intermediate catch tentacle is a permanent structure that appears identical to a fully mature catch tentacle. It is blunt-tipped, wider than adjacent feeding tentacles, and opaque (Fig. 1e). A regressing catch tentacle gently tapers to a pointed tip, like a feeding tentacle, but retains opacity (reminiscent of a catch tentacle) in distal tentacle regions (Fig. 1f).

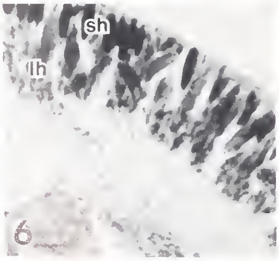


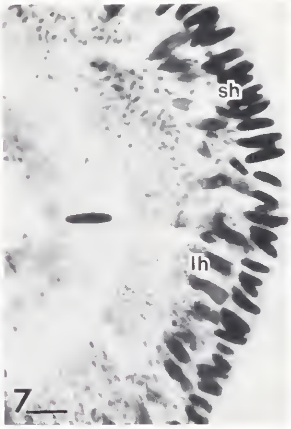












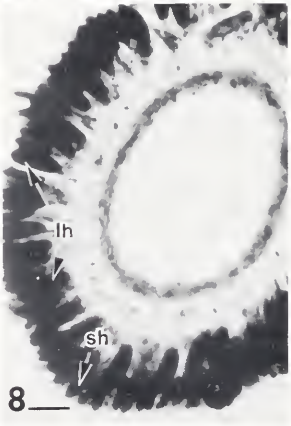


FIGURE 1. Illustration of catch tentacle developmental stages: (a) innercycle feeding tentacle (arrow), (b) stage 1 intermediate catch tentacle, "multiple-bulb" morph, (c) stage 1 intermediate catch tentacle, "single-bulb" morph, (d) stage 2 intermediate catch tentacle, (e) stage 3 intermediate catch tentacle (=mature catch tentacle), (f) regressing catch tentacle.

FIGURE 2. Photomicrograph of a feeding tentacle tip (formalin fixed and stained in azure a, eosin b). Note translucent spirocysts (s) and opaque microbasic p-mastigophores (m). Scale bar is 10 μm.

FIGURE 3. Photomicrograph of a stage 1 intermediate catch tentacle tip in cross section dominated by spirocysts (s). Scale bar is 10 μm.

Tentacle tip histology

Feeding tentacle

Feeding tentacles were dominated by spirocysts, followed by microbasic p-mastigophore nematocysts (Fig. 2). Occasional basitrich nematocysts also were present in these sections. The numerous mature cnidae were distributed at the epithelial surface while the occasional cnidoblasts were beneath them. Gastrodermal cnidae were rare in feeding tentacles.

Stage 1 intermediate catch tentacle

Stage 1 tentacles (histologically similar to feeding tentacles) were dominated by spirocysts, followed by microbasic p-mastigophores, and basitrichs (Fig. 3). Occasional cnidoblasts were observed in these tentacles along with a few gastrodermal cnidae.

Stage 2 intermediate catch tentacle

Stage 2 tentacles, although morphologically identical, varied histologically (Figs. 4, 5). Therefore, the tentacles were grouped into three characteristic substages (a, b, and c), which are described below.

Stage 2a tentacles, like feeding and stage 1 tentacles, were dominated by spirocysts, microbasic p-mastigophores, and basitrichs. However, the numbers of these cnidae were greatly reduced from those in feeding and stage 1 tentacles. Cnidoblasts were rare in these tentacles, but gastrodermal cnidae were more common than in feeding tentacles and stage 1 tentacles. Since 2a tentacles were qualitatively similar to stage 1 and feeding tentacles, a 2a tentacle photomicrograph is not shown.

Stage 2b tentacles were characterized by numerous cnidoblasts in their epithelium along with a few typical catch tentacle cnidae (small holotrich and large holotrich nematocysts), which appeared for the first time in this stage (Fig. 4). Feeding tentacle cnidae (spirocysts, microbasic p-mastigophores, and basitrichs) were rare in stage 2b tentacles. This is in contrast with feeding, stage 1, and stage 2a tentacles, in which feeding tentacle cnidae dominated the tentacle cnidom. However, gastrodermal cnidae were as common as in stage 2a tentacles.

Stage 2c intermediate catch tentacles were dominated by mature catch tentacle cnidae (small holotrich nematocysts), but unlike stage 2b tentacles, 2c tentacles

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FIGURE 4. Photomicrograph of a stage 2b intermediate catch tentacle tip seen in cross section. Note the numerous cnidoblasts (cb) in the tentacle epithelium and gastrodermal cnidae (gc) free in the tentacle coelenteron. Scale bar is 10 μm.

FIGURE 5. Photomicrograph of a stage 2c intermediate catch tentacle tip seen in cross section. Small holotrich nematocysts (sh) are distributed at the epithelial surface and large holotrichs (lh) are recessed from the epithelial surface. The tentacle is collapsed about its coelenteron. Scale bar is 10 μm.

FIGURE 6. Photomicrograph of a stage 3 intermediate catch tentacle tip seen in cross section. Small holotrichs (sh) line the epithelial surface and large holotrichs (lh) are recessed from the epithelial surface. Scale bar is 10 μm.

FIGURE 7. Photomicrograph of a mature catch tentacle tip seen in cross section. Note the numerous small holotrichs (sh) at the epithelial surface and large holotrichs (lh) recessed from the epithelial surface. Scale bar is 10 μm.

FIGURE 8. Photomicrograph of a regressing catch tentacle tip seen in cross section. Large holotrichs (lh) line the epithelial surface alongside small holotrichs (sh). Scale bar is 10 μm.

Note that Figures 2 through 8 are shown at the same magnification, indicating actual differences in tentacle size.

lacked cnidoblasts (Fig. 5). Feeding tentacle cnidae were rare in the 2c tentacle epithelium, but gastrodermal cnidae were often as common as in 2a and 2b tentacles.

Stage 3 intermediate catch tentacle

Stage 3 catch tentacles had many more cnidae than any of the types of tentacles discussed above (Fig. 6), because of the appearance of additional small holotrichs in these tentacles along with many large holotrichs (which had been extremely rare in stage 2 catch tentacles). The small holotrichs were distributed at the epithelial surface and the large holotrichs were recessed from the epithelial surface beneath the small holotrich cnidocytes. Feeding tentacle cnidae were extremely rare in stage 3 tentacles, as were gastrodermal cnidae. Cnidoblasts were absent from this stage. Note that the diameter of the stage 3 catch tentacle is much larger than that of the previous developmental stage (*i.e.*, significant growth has occurred).

Mature catch tentacle

Mature catch tentacles, like stage 3 catch tentacles, were characterized by numerous small holotrichs at the epithelial surface, followed by large holotrichs recessed from the epithelial surface (Fig. 7). However, feeding tentacle cnidae (rare in stage 3 tentacles) were absent from mature catch tentacle tips. Likewise, cnidoblasts were absent, and gastrodermal cnidae were rare in mature catch tentacles.

Regressing catch tentacle

Even though regressing catch tentacles had far fewer cnidae in their epithelium than mature catch tentacles, numerous small holotrich and large holotrich nematocysts were present in these tentacles (Fig. 8). Note that large holotrichs were distributed alongside small holotrichs at the epithelial surface in regressing catch tentacles. This holotrich distribution is in contrast with stage 3 and mature catch tentacles, in which large holotrichs were recessed from the epithelial surface. In addition, occasional feeding tentacle cnidae were observed in the tips of regressing catch tentacles, whereas none had been present in mature catch tentacle tips. Cnidoblasts were absent and gastrodermal cnidae were rare. Note that the diameter of the tentacle has decreased dramatically (*i.e.*, degrowth has occurred).

Quantitative histology

Feeding tentacle

Feeding tentacles had an average of 114.7 cnidae per tentacle tip cross section (equivalent to a 1.00 unit cnidom, n = 6). Of these 114.7 cnidae, 57.3% were spirocysts, 26.5% were microbasic p-mastigophore nematocysts, 5.2% were basitrich nematocysts, 2.2% were gastrodermal cnidae, and 9.8% were cnidoblasts (Fig. 9). The *H. luciae* feeding tentacle complement of spirocysts (at 57%) of the tentacle cnidom is lower than those reported by Purcell (1977) for *Metridium senile* (at 80%) and Schmidt (1982) for *Anemonia sulcata* (at 68%) but is in general agreement with the findings of Bigger (1982) for four anemone species. In this study, Bigger reported that the spirocyst complement ranged from 49% to 79% of the feeding tentacle cnidom.

Stage 1 intermediate catch tentacle

Stage 1 intermediate catch tentacles had slightly fewer cnidae than feeding tentacles with an average of 104.6 cnidae per tentacle cross section (104.6/114.7 = a

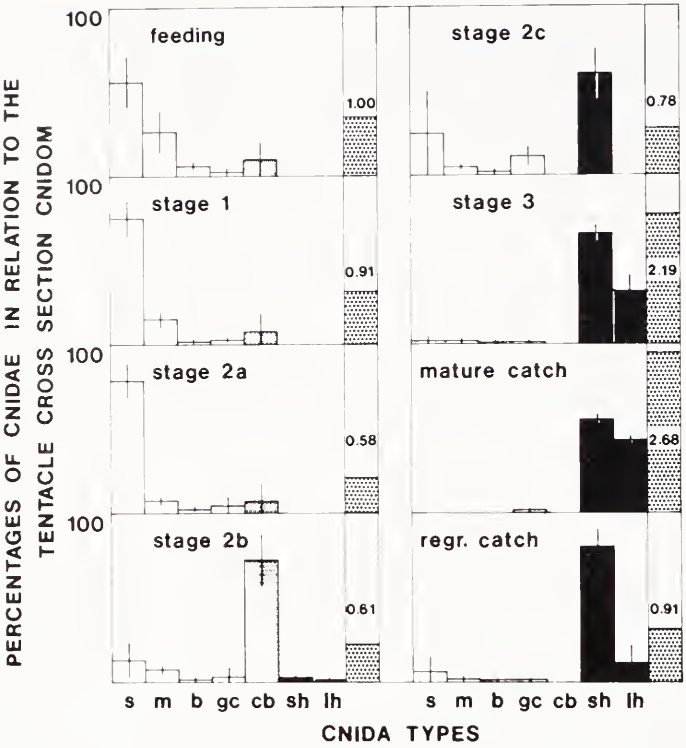

FIGURE 9. Percentages of cnidae in relation to the tentacle cross section cnidom. The percentage of each cnida type (listed below) was determined in relation to the tentacle cross section cnidom. These values were averaged for tentacles of the same type and the resultant values are shown in bars drawn to scale ± standard deviation (depicted as error bars). Feeding tentacle cnida types (white bars): spirocysts (s), microbasic p-mastigophores (m), basitrichs (b), gastrodermal cnidae (gc). Cnidoblasts (stippled bars, open circles) (cb). Catch tentacle cnida types (black bars): small holotrichs (sh), large holotrichs (lh).

In the inset at right (stippled bars, closed circles), the cnidom size is given for each type of tentacle (drawn to scale) as a function of the average total number of cnidae per feeding tentacle cross section (the unit cnidom).

0.91 unit cnidom, n = 3). The stage 1 intermediate catch tentacle cnidom was divided into 76.3% spirocysts, 14.3% microbasic p-mastigophores, 0.7% basitrichs, 1.7% gastrodermal cnidae, and 7.0% cnidoblasts (Fig. 9).

Stage 2 intermediate catch tentacle

Stage 2a tentacles were marked by a sharp decrease in the number of cnidae per tentacle section from the 0.91 unit cnidom of stage 1 tentacles to a 0.58 unit cnidom in stage 2a tentacles (n = 2). This "smaller" tentacle cnidom was made up of 78.3% spirocysts, 7.7% microbasic p-mastigophores, 2.7% basitrichs, 4.7% gastrodermal cnidae, and 6.7% cnidoblasts (Fig. 9). Note that all of the types of cnidae that occurred in feeding and stage 1 tentacles were present in stage 2a tentacles in percentages that were similar to those observed for stage 1 tentacles. However, since there was a sharp decrease in the cnidom size from 0.91 to 0.58, the decrease in numbers of cnidae was evenly distributed among all of the cnida types.

Stage 2b tentacles had a cross section cnidom (a 0.61 unit cnidom, n = 2) that was slightly greater than the 2a tentacle cnidom. The 2b tentacle cross section cnidom was composed of 13.0% spirocysts, 6.5% microbasic p-mastigophores, 0.5% basitrichs, 3.0% gastrodermal cnidae, 73.0% cnidoblasts, 3.5% small holotrichs, and 0.5% large holotrichs (Fig. 9). Thus, the percentages of feeding tentacle cnidae in general, and spirocysts in particular, decreased in 2b tentacles, while the percentage of cnidoblasts increased dramatically. However, the cnidom size of 0.61 unit cnidom was about the same as for the 2a tentacles. In addition, catch tentacle cnidae (small and large holotrichs) appeared for the first time in the epithelium of 2b tentacles.

Stage 2c tentacles had a cross section cnidom that was somewhat larger at a 0.78 unit cnidom (n = 2) than the previous two substages. The 2c intermediate catch tentacle cnidom consisted of 25.5% spirocysts, 4.5% microbasic p-mastigophores, 1.0% basitrichs, 11.0% gastrodermal cnidae, 0.0% cnidoblasts, 58.0% small holotrichs, and 0.0% large holotrichs (Fig. 9). Hence, 2c tentacles were characterized by the sharp decrease of cnidoblasts (to zero) along with a sharp increase in the percentage of small holotrichs. However, the percentage of large holotrichs did not increase in 2c tentacles, and the percentages of feeding tentacle cnidae remained low.

Stage 3 intermediate catch tentacle

The cnidom size of stage 3 catch tentacles (at a 2.19 unit cnidom, n = 7) was 2.8 times larger than that of stage 2c catch tentacles. The stage 3 catch tentacle cnidom was made up of 1.7% spirocysts, 0.9% microbasic p-mastigophores, 0.1% basitrichs, 0.3% gastrodermal cnidae, 0.0% cnidoblasts, 65.8% small holotrichs, and 31.0% large holotrichs (Fig. 9). Thus, this "large" tentacle cnidom was dominated by catch tentacle cnidae while each of the feeding tentacle cnida types (spirocysts, microbasic p-mastigophores, and basitrichs) decreased to less than 2.0% of the cnidom. These data are in general agreement with those of Purcell (1977) for newly developed catch tentacles (=stage 3 catch tentacles) of *M. senile.*

Mature catch tentacle

Mature catch tentacles had a larger cnidom size (at a 2.68 unit cnidom, n = 6) than stage 3 catch tentacles (at 2.19). The mature catch tentacle cross section cnidom comprised 0.0% spirocysts, 0.0% microbasic p-mastigophores, 0.0% basitrichs, 0.2% gastrodermal cnidae, 0.0% cnidoblasts, 56.2% small holotrichs, and 43.7% large holotrichs (Fig. 9). Note that the percentage of large holotrichs increased and the percentage of small holotrichs decreased in mature catch tentacles from those in stage 3 catch tentacles. Thus, the increase in cnidom size was caused by the addition of large holotrichs, while the number of small holotrichs stayed constant. This pattern is also shown by a comparison of the average raw numbers of these cnidae in mature catch tentacles versus stage 3 catch tentacles. An average of 170.2 small holotrichs and 137.5 large holotrichs were present in mature catch tentacles (per tentacle tip section), while 163.7 small holotrichs and 82.6 large holotrichs occurred in stage 3 catch tentacles.

Regressing catch tentacle

The cnidom size of regressing catch tentacles (at a 0.91 unit cnidom, n = 3) was about one-third that of mature catch tentacles. The regressing catch tentacle cnidom was made up of 6.0% spirocysts, 2.0% microbasic p-mastigophores, 0.3% basitrichs, 1.0% gastrodermal cnidae, 0.0% cnidoblasts, 80.3% small holotrichs, and 10.3% large holotrichs (Fig. 9). Therefore, at this point in catch tentacle regression, catch tentacle cnidae still dominated the cnidom even though their numbers were reduced by two-thirds. Notice that feeding tentacle cnidae had appeared in these tentacles in their "normal" relative proportions (*i.e.*, with spirocysts more numerous than microbasic p-mastigophores, which were more numerous than basitrichs). It is also important to point out that the percentage of gastrodermal cnidae was low (at 1.0%) in regressing catch tentacles. The significance of this "small" gastrodermal cnidae complement will be discussed later.

DISCUSSION

Catch tentacle development

Stage 1 intermediate catch tentacle

The multiple-bulb and single-bulb stage 1 tentacle morphs are each consistently observed in tentacles that develop into catch tentacles (the multiple-bulb morph appears first). Although nothing is known about the role of these bulbs in anthozoans, similar phenomena have been reported in hydrozoans, where they have been correlated with certain growth processes such as body lengthening and broadening. Beloussov (1973) studied "growth pulse" phenomena in stolon and hydranth growth in some hydrozoans, including *Dyamena pulima*. He thought that growth pulses arose from antagonistic myoepithelial cell movements between the hydrotheca and connective tissue layer that allowed the myoepithelial cell layer to extend beyond the hydrotheca, and thereby caused the stolon to elongate and the stolon tip to broaden.

Campbell (1980) recently proposed a model that related growth pulse phenomena to morphogenetic changes along *Hydra* stalks. Campbell suggested that myoepithelial cells stretch to their maximum extension by creeping of their cell processes in opposite directions. Continued creeping of these myoepithelial cell processes compresses the connective tissue layer. Hydrostatic pressure is generated in the gastric cavity that counteracts this compression of connective tissue and thereby deforms the myoepithelial cell layer. As a result, myoepithelial cells shift with respect to one another, and thus morphogenetic changes result. Campbell postulated that such processes are also involved in *Hydra* tentacle growth and development.

The resemblance borne by *H. luciae* stage 1 tentacle "pulses" (=bulbs) to known hydrozoan "growth pulses" suggests that these anthozoan tentacles might be involved in processes that account for tentacle widening and tentacle tip broadening during catch tentacle development, since catch tentacles are about twice as wide as feeding tentacles and blunt-tipped, whereas feeding tentacles are thin and have pointed tips.

This idea is supported by our observation of occasional multiple-bulb tentacle morphs in small tentacles in the outermost cycles of tentacle in some animals and also in small, newly-formed tentacles over fission scars. Thus tentacle "bulbs" similar to those in stage 1 intermediate catch tentacles occur in anemone tentacles that are almost certainly undergoing growth and development. Therefore, outer cycle "bulbed" tentacles are probably involved in general growth processes while inner cycle "bulbed" tentacles (which are already fully formed) are probably involved in the morphogenetic conversion of feeding tentacles into catch tentacles. Catch tentacles only develop in inner cycles of tentacles on the oral disc (Williams, 1975).

Inasmuch as the histology of stage 1 tentacle tips was similar to that of feeding tentacle tips, it is clear that stage 1 tentacles are not involved in cnidae turnover. This occurs in stage 2 tentacles and is discussed below.

Stage 2 intermediate catch tentacle

Feeding tentacle cnidae (spirocysts, microbasic p-mastigophores, and basitrichs) migrate (or are phagocytized by granulocytes and then transported) from the epithelium to the coelenteron in stage 2 tentacles. Hence, feeding tentacle cnidae are gradually removed from developing catch tentacles, but their fate beyond this point is not known. Perhaps these so-called "gastrodermal cnidae" are inserted into other anemone tissues (*e.g.*, into other feeding tentacles) or somehow eliminated and/or expelled from the anemone. The "removal" of feeding tentacle cnidae is followed by the appearance of numerous cnidoblasts in the epithelium. It is possible that interstitial cells (stem cells) migrate into stage 2 tentacles and then differentiate into cnidoblasts or, alternatively, that local stem cells (already present) proliferate and then differentiate into cnidoblasts. Since stage 2 tentacles were filled with numerous small holotrich cnidoblasts, or with numerous small holotrich cnidocytes, and not a mixture of the two, it is clear that the cnidoblasts synchronously mature into catch tentacle cnidocytes (the mature cell containing the mature structures).

Stage 3 intermediate catch tentacle

Large holotrichs are usually absent from the tips of stage 2 intermediate catch tentacles (which can contain numerous small holotrichs), and first appear in the tips of stage 3 intermediate catch tentacles. However, stage 3 catch tentacles consistently have fewer large holotrichs in their epithelia than mature catch tentacles (although stage 3 catch tentacles have about as many small holotrichs as mature catch tentacles). Thus, the appearance of large holotrichs occurs much later during catch tentacle development than the appearance of small holotrichs. These data suggest that the differentiation of interstitial cells into large holotrich cnidocytes is regulated so that the interstitial cells do not form into large holotrich cnidocytes until after many small holotrich cnidocytes have been produced and line the epithelial surface (*i.e.*, late in catch tentacle development).

Since stage 3 catch tentacles lack cnidoblasts at the tentacle tip, the source of the additional cnidae that are necessary for final catch tentacle maturation is unknown. Perhaps the pulse-like appearance of cnidoblasts and synchronous maturation into holotrich cnidocytes (observed in stage 2 tentacles) recurs throughout catch tentacle development. On the other hand, holotrichs might be produced in proximal tentacle regions and then transported through the tentacle coelenteron to be inserted into the distal tentacle epithelia. Another possible explanation is based on the fact that stage 3 catch tentacles, like mature catch tentacles, are functional, aggressive structures that autotomize their tentacle tips during normal functioning. Thus, this "deficiency" in the number of cnidae per tentacle section might be overcome by tissue renewal processes in the "new" catch tentacle tips following tentacle tip autotomy during aggression.

Mature catch tentacle

Note that the percentages of small and large holotrichs (respectively) in relation to the cross section cnidom were nearly identical among the six mature catch tentacles used in this study (*i.e.*, the standard deviation was small—see Figure 9). Since the mature catch tentacle cnidom is made up almost entirely of small and large holotrichs, it is possible that the total number of large holotrich cnidocytes that differentiate from interstitial cells in the latter stages of catch tentacle development is related to the total number of small holotrich cnidocytes that were produced in "early" stages of catch tentacle development (*i.e.*, in a fixed ratio of 1.0 large holotrichs produced per 1.3 small holotrichs). However, since the distribution of the two types of holotrichs is spatially distinct, with small holotrichs lining the epithelial surface and large holotrichs occupying the space beneath them, this ratio of large to small holotrichs might simply reflect optimal densities of each cnida type independent of the other. Catch tentacles are particularly suited for demonstrating possible regulation of this type (or maximization of space utilization) because normal tissue depletion in catch tentacles includes tentacle tip autotomy (an intermittent, all-or-none phenomenon).

Catch tentacle regression

The removal of catch tentacle cnidae (small and large holotrichs) from regressing catch tentacles is concurrent with the appearance of feeding tentacle cnidae. The holotrichs are not transported to the tentacle coelenteron (to become "gastrodermal cnidae") during catch tentacle regression, as feeding tentacle cnidae are during catch tentacle development. Instead, large holotrichs move from the middle epithelium to the epithelial surface alongside the small holotrichs, indicating that both types of holotrichs are probably expelled from these tentacles externally. The source of the mature feeding tentacle cnidae that appear in the tips of regressing catch tentacles is not yet known, since cnidoblasts were absent from the tips of the regressing catch tentacles used in this study.

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GROWTH AND SHELL VARIATION IN THE TROPICAL EASTERN PACIFIC INTERTIDAL GASTROPOD GENUS *PURPURA:* ECOLOGICAL AND EVOLUTIONARY IMPLICATIONS

GERARD M. WELLINGTON AND ARMAND M. KURIS[1]

University of Houston, Marine Science Program, 4700 Ave. U, Galveston, TX 77550, and [1]Marine Science Institute and Department of Biological Sciences, University of California, Santa Barbara, CA 93106

ABSTRACT

In this study we investigate variation in shell morphology among *Purpura pansa* Gould, 1853 and *P. columellaris* Lamarck, 1822; high intertidal predatory gastropods found in the eastern tropical Pacific. Field observations on the high frequency of shells intermediate in apertural characteristics, the occurrence of cross-copulations between distinct morph types, coupled with morphometric analyses and examination of sectioned shells lead us to conclude that these two forms are the same species. Hence, we propose *P. pansa* as a junior synonym of *P. columellaris.*

Growth studies reveal that the thicker shelled *columellaris* morph is much slower growing and represents a terminal growth stage of the *pansa* morph. Unlike some other thaidid species, starvation experiments failed to induce thickening of the apertural lip and apertural tooth formation. The stimulus that cues thickening is unknown.

Field experiments indicated that thick-toothed morphs are relatively more resistant to predation and are most common in wave protected, high predation risk environments. Absence of thick-shelled morphs in populations of the geminate congener, *P. patula* in the Caribbean suggests that predation is probably less severe in this region.

INTRODUCTION

Purpura columellaris Lamarck, 1822 and *P. pansa* Gould, 1853 (Family Thaididae), are predatory upper intertidal gastropods common on hard substrates in the tropical eastern Pacific. The northern geographic limit for both species is Baja California (Keen, 1971). Peña (1975) records *P. pansa* from northern Peru; Keen (1971) lists *P. columellaris* further south, from Chile. Both species occur in the Galápagos Islands. *Purpura pansa* is similar to its germinate species in the Caribbean, *P. patula* and previously has been regarded as a subspecies of the latter (Clench, 1947). There is no counterpart of *P. columellaris* in the Caribbean.

The two co-occurring forms are identified by several distinguishing features. The submarginal apertural teeth, a thickened outer lip and a tooth on the columella, characterizes *Purpura columellaris,* whereas *P. pansa* has a thin, often crenulated outer lip and lacks apertural and columellar teeth (Clench, 1947). In Panamá and the Galápagos Islands, one of us (GMW) commonly observed intermediate forms. Lowe (1932) also found intermediate shells.

In this paper we quantify and compare morphometric variation in these two species including a description of the intermediate specimens. Through observations

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of copulatory behavior, habitat, growth in the field and the nature of the morphometric variation, we conclude that the thick-shelled, *P. columellaris* is a terminal, but nonobligate slowly growing form of *P. pansa.* Since *P. columellaris* has priority, we propose *P. pansa* as a junior synonym. Second, we provide evidence to suggest that changes in shell shape and growth rate may be an adaptive response to intense predation pressures. Lastly, we speculate on the lack of a thick-shelled growth form for *P. patula,* the Caribbean amphi-species of *P. pansa.*

MATERIALS AND METHODS

Field sites and sampling techniques

Purpura are predaceous thaidid gastropods which live in the mid to upper intertidal zone. Foraging and reproductive activities (*i.e.* copulation and ovipositing) occur in crevices during periods of low water (Garrity and Levings, 1981).

Field work was carried out at two localities: Academy Bay, Santa Cruz Island, Galápagos, Ecuador and the Perlas Islands (Chitre and Saboga), Panamá. Densities and frequencies of shell types were gathered by sampling 0.5 m each side along line transects (n = 15, 5–10 m in length) laid out parallel to the shoreline across the high and mid intertidal zones. Size-frequency distributions were collected on shells at the Panamá sites. Shell types were noted, and total shell length measured using vernier caliphers.

Frequencies of cross-copulations between individuals with shells typical of *P. pansa* and *P. columellaris,* (*i.e.* excluding intermediates) were recorded at both the Galápagos and Panamá sites over a period of several months. Copulating individuals were temporarily detached from the substrate and shell type and sex were noted. In the Galápagos, copulation occurs in every month of the year, similar to the situation observed in Panamá (Garrity and Levings, 1981).

Shells used in the morphometric analyses (see below) were collected from several islands in the Galápagos. For these analyses an attempt was made to randomly collect shells over a wide range of sizes and shell types.

Morphometric analyses

To evaluate differences in shell shape between these nominate species of *Purpura* we subjected our shells to a principal components analysis (PCA). Prior to measurement each shell was determined to be *P. pansa* or *P. columellaris* according to lip thickness and the presence of teeth. Intermediate shells (Fig. 1) were grouped into three classes based on their similarity to *P. pansa* or *P. columellaris.* Intermediate class one shells were similar to *P. pansa* but laminar deposition had submarginally thickened the apertural lip. Emergent teeth were present on either the apertural lip or the columella. Class two shells had emergent teeth on both the columella and the lip. Class three shells resembled *P. columellaris* but the teeth were not as prominently developed and the last portion of the body whorl was not as heavy as typical *P. columellaris.* Since a complete series of intergrades was available our criteria for distinguishing classes were necessarily subjective.

A sample of 273 shells from the Galápagos Islands were included in the principal components analysis. For each shell, six variates were measured. Length was the distance from the tip of the spire to the anterior end of the shell. Spire height was the distance from the top of the spire to the origin of the suture at the apertural margin. Width was the maximum measurement at a right angle to length along the axis. Apertural length was measured parallel to shell length, and aperture width was

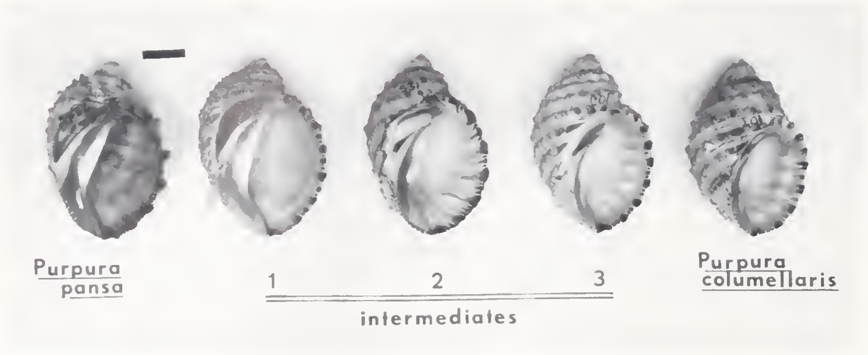

FIGURE 1. *Purpura pansa, P. columellaris* and shells of intermediate diagnostic morphology (see text). Dark bar = 1 cm.

the greatest distance from the margin of the outer lip to the columella measured perpendicular to aperture length. Dry weight of the shell was the sixth variable. Linear measurements were taken with vernier calipers to 0.1 mm; weights on an analytical balance to 0.01 g.

For the PCA all measurements were transformed to natural logarithms, and all variates were normalized. These procedures satisfy the assumption of PCA that all samples be drawn from a multivariate, normal distribution (Blackith and Reyment, 1971).

Principal components analysis associates each shell with a point in a 6-dimensional space where each dimension represents one variable. Any line through the origin in this space is a new synthetic variable that gives to any shell a value equal to the distance from the origin to the perpendicular projection of that shell onto that line. The line that removes the most variance is the first principal component (PC I). The line perpendicular to PC I that accounts for the largest remaining variance is the second principal component, and so forth. All six components are uncorrelated. The proportion of the variance subsumed by each component is expressed as an eigenvalue. Interpretation of the principal components is achieved by examination of the eigenvectors. This provides a series of loadings showing the correlation of the original variables with the principal components (Blackith and Reyment, 1971; Kuris and Brody, 1976).

The multivariate statistics indicated that spire height in relation to shell size differed for *P. pansa* and *P. columellaris.* We performed analyses of covariance (ANCOVA) for log spire height on log shell length. For a subset of 20 *P. pansa* and *P. columellaris* we compared spire height with a seventh shell measurement, old spire height, using regression analysis and a nonparametric test of the residuals. Old spire height was the distance from the tip of the spire to the suture of the body whorl 180° opposite the posterior notch of the aperture.

To reveal the history of past growth of these shells, sections were cut with a rock saw. Sections from 25 shells showed the sequential deposition and relative extent of prismatic and laminar shell layers as well as patterns of abrasion and the laminar deposition of shell from the body surface in repair processes.

To test our classification of *Purpura* shells we performed several discriminant functions analyses (DFA) on our morphometric data. A discriminant functions analysis is a very robust multivariate classification procedure because discriminant functions are derived so as to maximize group differences. Examination of the proportion and nature of misclassifications in the several classification schemes permits us to reject some proposed taxonomic groupings. A discriminant function analysis weights and linearly combines the six shell variables so that the groups identified on an *a priori* basis are forced to be as statistically distinct as possible. Since the initial variables are assumed to be drawn from a multivariate normal distribution, our data were logarithmically transformed and normalized before proceeding with the DFA (Klecka, 1975). Our *a priori* determination of shell group membership used the same combinations of apertural tooth development and lip thickness discussed above. Statistics of the discriminant functions analysis were generated by the SPSS (Statistical Package for the Social Sciences) program (Klecka, 1975). All other statistics were performed with SAS (Statistical Analysis System; Ray, 1979) statistical programs.

Determination of growth rate and starvation experiments

To test the hypothesis that *P. columellaris* represents a terminal growth stage of *P. pansa* we measured growth rates (*i.e.* lateral extension of the outer lip) of *P. pansa* and *P. columellaris.* At the southwest corner of Saboga Island, Panamá, fifteen snails of *P. pansa* (including intermediate class 1) and *P. columellaris* (including intermediate class 3) were marked by file-etching a small groove (2–3 mm deep) into the growing edge of the outer lip. One or more grooves were made on each shell in a unique and identifiable pattern. The animals were released and recovered five months later (26/1/79 to 13/6/79). Extension of the aperture past the groove markings was measured using dividers and a rule.

To test whether starvation could induce shell thickening in *P. pansa,* cages were used to enclose animals *in situ.* Three small cages (20 × 20 × 5 cm) constructed of plastic coated wire frame (2 × 6 cm) and covered with 1 cm wide Vexar® mesh were attached with screws and epoxy to a vertical rock wall within the natural habitat near the interface between the high and mid tide level. One, three, and six *P. pansa* were marked and placed within the three cages respectively. Five *P. pansa* were etched and left outside the cages as controls on adjacent rocks. The experimental period ran from 16/6/79 to 20/11/79.

Predation experiments

To examine the adaptive significance of shell polymorphism we investigated the relative susceptibility of thin (*P. pansa*) and thick (*P. columellaris*) shells to predation. Shells (without animals) were attached with epoxy to open rock surfaces at the high-mid tide interface along both wave-exposed and protected shorelines on Chitre Island, Panamá. Shells were attached during low tides in November 1979 and left for 48–96 hours.

RESULTS

Habitat and population structure

Purpura were most abundant in areas exposed to moderate wave action. Mean population density at the Academy Bay (Galápagos) site was 0.69 individuals/m^2 (area sampled = 100 m^2). Densities were variable; highest (>2 individuals/m^2) in or near crevices and under boulders and lowest (0/m^2) on exposed flat rock surfaces. Similar results concerning microhabitat distribution have been reported by Garrity and Levings (1981) in Panamá.

Surveys in wave exposed areas indicated that *P. pansa* was the more common species, accounting for 70% of the population (Table I). Ten percent of the snails were intermediate in shell morphology between *P. pansa* and *P. columellaris* (see Fig. 1).

While *P. pansa* was the more abundant species, size frequency data show that *P. columellaris* was significantly larger (Fig. 2: median size = 40 mm for *P. pansa,* 49 mm for *P. columellaris;* $P < .05$ Mann Whitney U-test). A few *P. columellaris* less than 30 mm in length were found during this study (smallest was 20 mm), however, the smaller size classes were predominated by *P. pansa.*

Cross-copulations

Purpura moves out of crevices on falling tides to forage and engage in reproductive activities. During copulation the male climbs onto the shell of the female near the posterior end and inserts its penis into the copulatory bursa located just beneath the mantle (Fig. 3). When disturbed, the penis is withdrawn slowly confirming that copulation actually occurred. In almost all cases males were considerably smaller than females, however, we found no correlation between sex and shell morphology.

Cross-copulations between distinct shell morphs (*i.e. P. pansa* × *P. columellaris*) were not uncommon (Table II) nor were copulations involving intermediate shell forms with one or the other of the distinct morphs (Fig. 3). We have no evidence that these cross-copulations result in viable offspring. Nonetheless, these observations strongly suggest that *P. pansa* and *P. columellaris* are not behaviorally isolated from reproducing. Cross-copulations however, do occur less frequently than expected based on the proportion of *P. pansa* and *P. columellaris* morphs in the population (Table II). Microhabitat differences in the distribution of the morphs may decrease opportunities for cross-copulations.

Morphometric analyses

A covariance matrix for the sample of 273 shells disclosed that all six variables were highly correlated (r ranging from 0.803 to 0.990). Not unexpectedly, the eigenvector of principal component I (Table III) yielded loadings of the shell measurements with PC I that were all highly positive correlations of similar value (r ranging from 0.394 to 0.420). Thus, we interpret PC I to be a synthetic variable representing overall size in accord with other morphometric studies of arrays of similar objects of different size (Blackith & Reyment, 1971; Kuris & Brody, 1976). Most of the variance in the original data set was described by PC I (94.0%). The remaining variance must then be attributed to factors other than size, *i.e.,* aspects

TABLE I

Frequency of occurrence of Purpura *morphs in the Galápagos Islands, Ecuador and in the Gulf of Panamá, Panamá*

Location	N	Thin, untoothed *P. pansa*	Thick, toothed *P. columellaris*	Intermediates (1, 2 and 3)
Galápagos	152	103	34	15
Panamá	110	79	22	9
Totals	262	182	56	24
%	—	69%	21%	9%

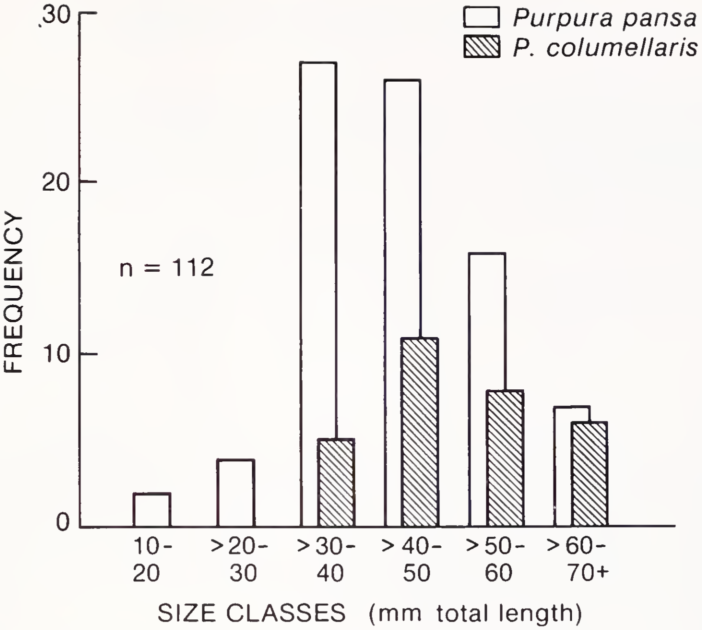

FIGURE 2. Histogram showing the size-frequency distribution of *Purpura pansa* and *P. columellaris* at Chitre Island, Panamá.

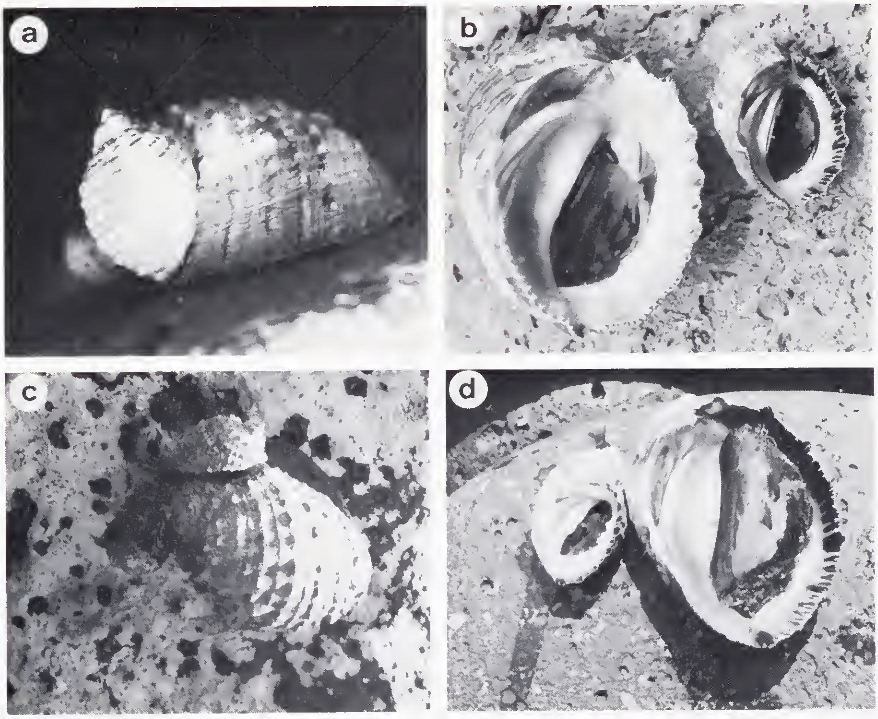

FIGURE 3. Field observations of cross-copulating individuals of *Purpura*. (a) copulation between *P. pansa* (small male) and an intermediate (class 2) morph (large female). (b) same individuals in (a) showing details of shell morphology. Emergent teeth on the outer lip of the aperture and columella are visible in the large female. (c), (d) copulation between *P. pansa* (large female) and *P. columellaris* (small male).

TABLE II

Frequency of copulations observed among and between morphologically distinct snails (i.e. thick, toothed P. columellaris *and thin, untoothed* P. pansa*) in the Galápagos Islands and at various island locations in the Gulf of Panamá*

		Within-morph matings		Cross-copulations
Location	Total number of copulations	*P. pansa*	*P. columellaris*	**P. pansa* × *P. columellaris*
Galápagos	52	34	10	8
†Panamá	89	65	13	11
Totals	141	99	23	19
%	—	70	16	13

* Calculated expected frequencies of cross-copulations using the binomial distribution indicate that observed values are significantly less than expected based on the proportion of *P. pansa* and *P. columellaris* in the samples ($\chi^2 = 21.48$, $P < .005$, for Galápagos; $\chi^2 = 32.28$, $P < .005$, for Panamá, see text for discussion).

† A portion of these data represent unpublished observations made by S. Garrity and S. Levings and are cited here with their kind permission.

of shape. Principal component II accounted for 4.6% of the variance and therefore contained most of the information on shape. Spire height and weight gave strong negative correlations and aperture length and aperture width strong positive correlations with PC II (Table III). In visual terms (Fig. 4), shells having a high score on the second component tended to be low spired and light in weight. Their apertures were large (both wide and long). Figure 4 shows that *P. pansa* shells tended to have low spires, light weights and large apertures, whereas *P. columellaris* tended to be heavy, have high spires and small apertures. Thus PCA gives an incomplete separation of *P. pansa* and *P. columellaris.* Most of the intermediate shells do fall between the two species with a gradient along PC II of intermediate class 1 shells falling near *P. pansa* and intermediate class 3 shells positioned near *P. columellaris.* It is also quite evident from Figure 4 that most small shells are *P. pansa* type.

TABLE III

Eigenvectors and eigenvalues (expressed as proportion of the total variance) for the principal components analysis of 273 shells and 6 input variables, and for the PCA of 54 shells over a restricted range of sizes (see text)

	Principal components (273 shells)		Principal components (54 shells)	
Original variables	I	II	I	II
shell length	0.420	0.030	0.508	−0.198
spire height	0.394	−0.612	0.170	−0.589
shell width	0.419	0.136	0.526	−0.069
aperture length	0.413	0.337	0.506	0.272
aperture width	0.402	0.535	0.406	0.472
weight	0.402	−0.454	0.125	−0.595
Eigenvalue (%)	94.0	4.6	49.7	38.0

PC III to VI are omitted as they cumulatively included only 1.2% of the variance in the first analysis.

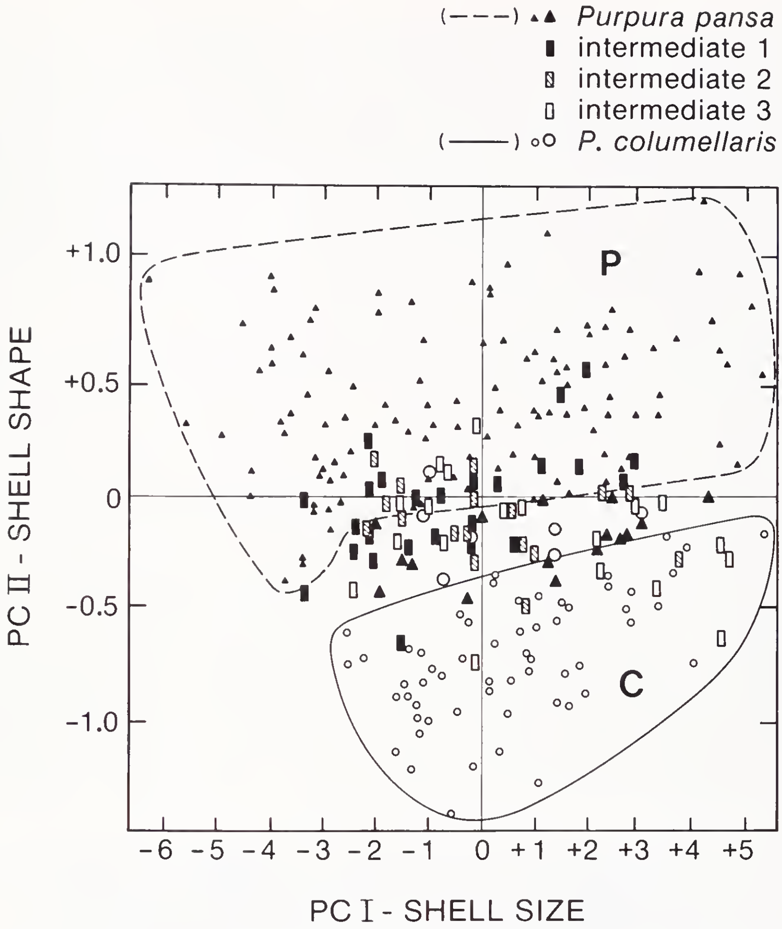

FIGURE 4. Plot of 273 shells used in PCA on the coordinates representing shell size (PC I) and shape (PC II) (see text for interpretation of these axes). All shells identified on the basis of apertural morphology as *Purpura pansa* are indicated by solid triangles and the region occupied by the majority of these shells has been demarcated by the dotted line. All *P. pansa* shells within this zone are designated by small triangles while those located outside the dotted line are indicated by large triangles. Similarly, *P. columellaris* shells are indicated by open circles and a solid line. Intermediate shells of types 2, 3 and 4 (see text) are designated by solid, diagonally shaded and open rectangles, respectively.

To further examine the variance apportioned along the shape components we repeated the PCA using a restricted range of shell sizes. This analysis included all 54 shells within the range of 0.0 to +1.25 on PC I in the original analysis. As Table III shows, the PC I could still be interpreted as a size related variable although spire height and weight now contribute little to PC I. PC II now included a substantial 38.0% of the variance. The loadings on the eigenvector for PC II were generally similar in magnitude and direction to PC II of the 273-shell PCA. Thus, this analysis confirmed that PC II separated shells on the relative dimensions of spire height and weight versus aperture size (both width and length). Examination of plots (not shown) again showed that *P. pansa* tended to have a large aperture, a low spire and are relatively light compared to *P. columellaris*.

Growth model

Field observations on size distributions and cross-copulation between *P. pansa* and *P. columellaris* shells, the presence of numerous shells with intermediate patterns of apertural morphology, and our PCA documentation that these shells intermediate in apertural sculpture were also morphometrically intermediate, suggests that these nominate taxa are growth forms of the same species. We hypothesize that all shells start growth as the *pansa* morph and some selectively thicken the distal portion of the body whorl and develop apertural teeth, thus transforming to the *columellaris* morph. Since no shells were observed to develop in the reverse direction we further propose that this change is unidirectional and irreversible.

Further support comes from an examination of shell sections (Fig. 5). The outer wall of shells with a *pansa* morph has a relatively thin laminar layer secreted by the mantle margin over a thin prismatic layer. The terminal portion of the outer wall (approximately the distal third of the body whorl) of all *P. columellaris* morphs has a thick layer of laminar shell deposited on a thin prismatic layer. However, in the older parts of *P. columellaris* shells only a thin laminar layer is present. The *columellaris* morphs resemble the *pansa* morphs with respect to the relative deposition of laminar material on the outer wall in older parts of the shell. Also evident in Figure 5 is the deposition of a laminar regenerative layer secreted by the mantle surface of the body (rather than by the free mantle surface which produces the laminar layer). This regenerative layer is apparently secreted in response to thinning of the shell due to external abrasion. On the very eroded, oldest portion of the spire this regenerative layer may completely replace the original prismatic and nacreous layers. A similar regenerative shell layer is present in the spire of many prosobranch molluscs. Details of the microstructural components of the regenerative layer has recently been described for *Tegula* by Geller (1982). Observations of sectioned shells

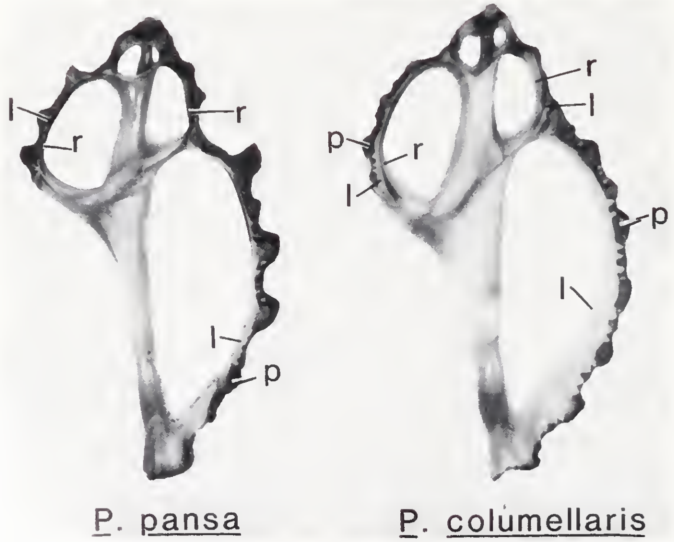

FIGURE 5. Sections of *Purpura pansa* shell (left) and *P. columellaris* shell (right). Sections were cut through the axis of the shell along the columella such that the right-hand side of the shell is about 1–2 mm from the aperture. Both shells were 45 mm long. Shell layers are designated p, prismatic layer; l, laminar layer; and r, regenerative layer.

suggest that increased deposition of this regenerative layer thickens the outer wall of the spire of the *columellaris* morph more extensively than for the *pansa* morph (Fig. 5).

The loadings of the eigenvector of PC II disclosed a morphometric difference between the *pansa* and *columellaris* morphs that had not been suspected *a priori* and suggested a test of this one species-two growth forms hypothesis. The postulated initial *pansa* growth form tended to have a large aperture (both long and wide) and consequently a short spire. This difference in relative spire height was confirmed by an ANCOVA of log spire height on log shell length for shells identified as *P. pansa,* the 3 intermediate classes and *P. columellaris* on the basis of apertural tooth development and body whorl thickness. No significant differences were found among slopes for all 5 shell types. Intercepts of both *pansa* and *columellaris* morphs were significantly different from each other and from all three intermediate classes. Grand means of the *pansa* morph had significantly lower spire heights than did the *columellaris* shells.

If growth proceeds to a *columellaris* morph, the aperture must exhibit a relative decrease in allometric growth resulting in a relatively high spire since aperture length and spire height are inversely proportional measurements. Thus, a meaurement of former spire height taken at a sufficient distance before the aperture along the body whorl of the *columellaris* morphs should be relatively small compared to the present spire height if growth of the aperture length of these shells has not kept pace with growth of the rest of the shell. In contrast, the old spire height of the *pansa* morph should be similar to the present spire height as a relative proportion of shell size. For this comparison we selected an old spire height 180° opposite the aperture. If the relative change in spire height is confirmed for the *columellaris* morph, this would further indicate that the change from the *pansa* to the *columellaris* morphs occurs over less than one half of a whorl and therefore that the *columellaris* morphs grow very slowly, if at all, once the transformation is complete. Present spire height of the *columellaris* shells is high relative to the old spire height (Fig. 6). All of the log-transformed values for previous versus present spire height in *P. columellaris* fall above the regression line for the *pansa* shells. This difference is significant (X^2, $P < .01$). A comparison of the *columellaris* and *pansa* old spire height-present spire height is permissible since the analysis of covariance of present spire height on shell length showed that allometry of the slopes was very similar (0.927 for *pansa* and 0.889 for *columellaris*, $P > .25$).

Classification tests

Principal components analysis and regression statistics support the hypothesis that *pansa* and *columellaris* morphs are growth forms of the same species. The intermediate shells represent specimens in the process of transforming from the former to the latter morph. Classification procedures in the descriminant functions analysis were used to inspect and test this as well as competing hypotheses. The first classification (Table IV) was based on a discriminant function that strongly separated shells determined on the basis of apertural teeth and lip thickness to be *P. pansa* and *P. columellaris,* respectively. Over ninety-nine percent of these shells were successfully discriminated by this procedure. However, when this discriminant function allocated the intermediate shells, 58 percent were assigned to *pansa,* 42 per cent to *columellaris.* This relatively even split would be expected if the intermediate shells represented a continuum from *pansa* to *columellaris.* Further support for this interpretation of the intermediate shells would be gained by a high likelihood of

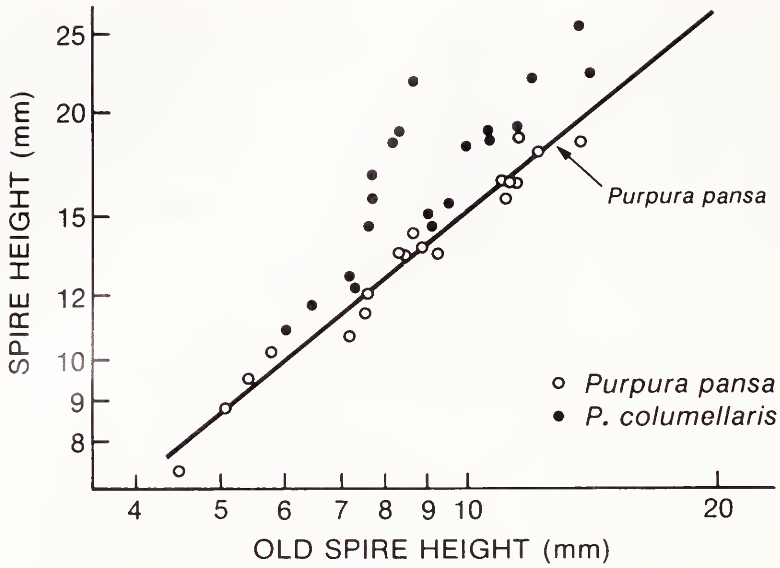

FIGURE 6. Relationship between log old spire height and log spire height (see text for definitions) of 20 shells identified as *Purpura pansa* (open circles) and *P. columellaris* (closed circles) on the basis of apertural morphology. The regression line was fitted to the data for *P. pansa* only to show the relative increase in spire height of *P. columellaris* following the growth of one-half of a body whorl.

misclassification. Such was reflected by a low probability (<.05) of being located as far as they were from either centroid. By this criterion, 48% of the intermediate shells could not be reliably classified.

TABLE IV

Discriminant functions analysis classification of shells following assignment of shell to predicted shell groups on the basis of apertural teeth and lip thickness

DFA	*a priori* classification	Percentage of shells correctly classified	Percentage of shells misclassified as P	I	C
1	P	99	—	—	1
	C	100	0	—	—
	ungrouped I*	—	58	—	42
2	P	87	—	12	1
	I	79	13	—	8
	C	92	0	8	—
3	P + I†	93	—	—	7
	C	98	(2)		—
4	P	97	—		(3)
	C + I†	88	12	—	—

P = *Purpura pansa*, I = intermediate, C = *P. columellaris*.

* In the first DFA the intermediate shells were not grouped and the classification procedure assigned each shell to either P or C based on their loadings on the discriminant function.

† In the third and fourth DFA, intermediate shells were combined with either P or C. Numbers in parentheses refer to the percentage of shells misclassified into the combined categories.

The second DFA tested the hypothesis that the intermediate shells represented a third taxon, distinct from either *pansa* and *columellaris* and about 10 per cent of each of these latter shell types were misclassified as intermediate. This pattern is not consistent with a hypothetical third type that is not intermediate.

The third and fourth DFAs jointly examine the possibilities that either *P. pansa* or *P. columellaris* are variable species with the intermediate shells being either "toothed" *P. pansa* (first hypothesis) or "smooth" *P. columellaris* (second hypothesis). Thus, a DFA was performed to separate *pansa* plus the intermediates from the *columellaris* shells (Table IV). Here a high degree of separation was achieved with only 5.5% of the shells misclassified. However, the same degree of separation was achieved in the reverse analysis (*columellaris* plus intermediates versus *pansa*) since only 7.7% were misclassified. Taken together, these tests enabled us to reject both of these "variable species" hypotheses.

Growth rates

Results from the morphometric analyses suggest that *P. columellaris* is a terminal growth form of *P. pansa*. This hypothesis predicts that the former morph should be slower growing than the latter. Growth measurements recorded at Saboga (Panamá) appear to confirm this prediction (Table V). Using similar sized snails, the apertural lip of *P. pansa* grew twice as fast as that of *P. columellaris* ($P < .01$, Mann Whitney U-test).

Starvation experiments

There is some evidence that thickening can be induced by stress such as starvation, in other thaidids (Bryan, 1969; Cowell and Crothers, 1970). Results from our enclosure experiments using *P. pansa* morphs indicate that at least severe starvation is not an important stimulus for shell thickening. We found no evidence of tooth formation among our experimentally caged snails. The apertural lip in several animals, however, had become quite worn. Shell morphology of all the control animals remained unchanged throughout the experiment.

Considering the slow growth of *Purpura* and the complete starvation conditions of our experiment we cannot reject the starvation hypothesis with confidence. It may be that chronic or periodic rather than complete starvation induces shell thickening.

Predation experiments

The distribution of *Purpura pansa* and *P. columellaris* generally overlap but often differ quantitatively depending on exposure to wave action. For example, at

TABLE V

Linear growth of the outer lip of Purpura pansa *and intermediate class 1 versus* P. columellaris *and intermediate classes 2 and 3 from 23 January 1979 to 15 June 1979 at the southwest corner of Saboga Island, Gulf of Panamá*

Shell form	Sample size	Total shell length (mm)	Linear growth of the outer lip after five months (mm)
P. pansa + intermediate 1	10	41.7 (±12.35)	2.7 (±.65)
P. columellaris + intermediates 2 and 3	5	41.5 (±8.25)	1.4 (±.55)

Values represent means with standard deviations in parentheses.

Chitre Island (Panamá) we found that in wave exposed situations (southern shore) 76% of the *Purpura* population was composed of *pansa* morphs while only 14% were *P. columellaris* and 10% intermediates (n = 99). Conversely, in protected area (northern shore) *Purpura* were much less abundant but were predominantly *P. columellaris* (73% *P. columellaris,* versus 27% *P. pansa:* n = 11).

Using tethered *Purpura* Garrity and Levings (1981) found that within a 24 hour period fish predation resulted in 25% mortality. Results from our experiments, placing thick (*P. columellaris*) and thin (*P. pansa*) shells in wave exposed and protected areas, reveal that thick shells were more resistant to shell breakage than were thin shells, particularly along protected shorelines (Table VI: on wave exposed shores difference in shell damage between thick and thin-shelled morphs was not significant, $P > .05$, but on protected shores thin-shelled morphs suffered significantly higher damage, $P < .01$, Fisher's Exact Test).

Because characteristic fused-tooth marks were found on the surfaces of several unbroken thick shells, we suspect that pufferfish (*Arothron* spp. and *Diodon* spp.) were responsible for most of the experimentally observed shell damage. Garrity (pers. comm.), however, has pointed out to us that crab predation may be an even greater source of differential mortality among *Purpura* shell morphs. Thickening of the apertural lip in gastropods has been shown to reduce successful predatory attacks by crabs (Vermeij, 1978).

DISCUSSION

Field observations on the high frequency of shells intermediate between *Purpura pansa* and *P. columellaris* in apertural characteristics, numerous observations of cross-copulation between these types, inability to locate very small *P. columellaris* in the field (or in museum collections), when coupled with our morphometric analyses and shell sections, strongly suggest that these two long recognized taxa are the same species. Thus, we propose that *P. pansa* Gould, 1853 is a junior synonym of *P. columellaris* Lamarck, 1822.

The *columellaris* morph of *P. columellaris* appears to be a facultative terminal growth form. All shells initially grow as the *pansa* morph. Transformation to the *columellaris* morph can occur over a very wide size range. This change includes the development of columellar and submarginal teeth in the aperture. The apertural lip

TABLE VI

Results of predation experiments on pansa *and* columellaris *morphs in the mid-intertidal zone at Chitre Island, Panamá*

Situation	Shell morph	Sample size	Mean total length-mm (range)	Number of shells damaged (%)	†Significance level
exposed	*pansa*	31	42.1 (35.7–48.7)	3 (9.6%)	
(southern shore)	*columellaris*	17	42.3 (36.1–51.5)	0 (0)	$P > .05$
protected	*pansa*	33	41.4 (35.0–47.1)	17 (51.5%)	
(northern shore)	*columellaris*	20	43.8 (33.6–51.0)	2 (10.0%)	$P < .01$

Experiments ran 48–96 hours.
† Fisher's exact test.

thickens. Linear growth rate decreases. Increased laminar deposition thickens the outer wall of the younger portion of the body whorl and increased regenerative layer deposition thickens the older portion of the shell. As the transformation proceeds, the relative growth of the aperture decreases to produce a high spired, small apertured shell. Transformation from the *pansa* to the *columellaris* morph is completed over the growth of less than half a whorl. The process appears irreversible since shell sections never disclosed early periods of tooth formation or increased laminar deposition on the shell wall. The *columellaris* morph probably grows very slowly since old spire height measurements were always relatively small. Vermeij (1980) also reports a strong inverse correlation between spire height and shell growth rates. Observations on growth in the field suggests that the *columellaris* shells are growing slower than *pansa* morphs of the same size. Growth of the *columellaris* shells did not cease since prismatic shell deposition was still evident at the apertural margin.

The only counter evidence to this evaluation is the record of *P. columellaris* from Chile (Keen, 1971). If this species is merely a facultative terminal growth form then its geographic range must be inclusive of the range of initial *pansa* growth form. Examination of extensive new material at the Los Angeles County Museum (LACM) reveals that *P. pansa* occurs south to Islas Lobos de Afuera, Peru, a record south of the locality, Piura, listed by Peña (1975) in his extensive checklist of Peruvian molluscs. Peña (1975) did not list *P. columellaris* from Peru. At LACM the southernmost collections of *P. columellaris* was La Libertad, Ecuador, where it occurred with the *pansa* morph. Finally, McLean (pers. comm.) who has collected extensively along the western coast of South America states that neither species occurs in southern Peru or Chile. Based on this new information the geographic data are consistent with our one-species, two growth form analysis. The record in Keen (1971) may be in error.

The development of apertural teeth during periods of starvation and slow growth has been reported for another thaidid gastropod, *Nucella lapillus* (Cowell and Crothers, 1970; Crothers, 1971). However, in this species the process is reversible. When conditions improve, relatively rapid growth is resumed and teeth are no longer secreted along the apertural margin. In these shells the presence of teeth inside the aperture marks past periods of slow growth. A similar pattern has been observed by AMK (pers. observ.) for *Acanthina punctulata* and for *N. lamellosa* by Spight *et al.* (1974). Temporary periods of arrested linear growth are associated with the development of thick ribs, varices and complex apertural sculpture in many snails (*e.g.* muricids, cassids, cymatiids) (MacKenzie, 1961; Laxton, 1970; Spight, 1973; Spight *et al.*, 1974). *Rissoa parva* exhibits a seasonal tendency to alternate between ribbed and smooth shell morphs (Wigham, 1975). In other snails growth ceases and a terminal morphology develops that typically includes increased laminar deposition forming a thickened apertural lip. Termination of growth is often obligatorily associated with the onset of sexual maturity. Examples include cypraeids, aporrhaids, cymatiids, strombids, helicids and *Cerithium nodulosum* (Randall, 1964; Laxton, 1970; Spight *et al.*, 1974; Pollard *et al.*, 1977; Yamaguchi, 1977; Kat, 1981).

The relationship between the morphs of *Purpura columellaris* has some relatively unusual features. The transformation is not reversible. It is not necessarily associated with sexual maturation and it is not obligatory. As indicated in Figure 4, switch to the *columellaris* morph occurs over a very wide range of sizes [as Yamaguchi (1977) shows also for *Cerithium nodulosum*] and many large specimens of the *pansa* morph are sexually mature. It remains to be confirmed whether sexually immature *columellaris* morphs exist. The cue for the transformation is presently unknown.

Variation in shell sculpturing and morphology has been postulated to be a response to one or more environmental conditions (Moore, 1934, 1936; Phillips *et al.*, 1973; Spight, 1973; Kitching and Lockwood, 1974; Wigham, 1975; and others). Evidence has been found for some species to indicate direct genetic control of variation and that patterns of distribution reflect differential mortality or habitat selection (Struhsaker, 1968). Little is known about the reproductive biology of *Purpura* except that S. Garrity and S. Levings (pers. comm.) have found gelatineous egg masses. We examined several small shells (<10 mm total length) and found evidence of an embryonic shell (protoconch I) occupying the first whorl and a second protoconch extending an additional one to two whorls. The presence of the second protoconch indicates a pelagic juvenile stage (Jablonski and Lutz, 1980). Thus, it is probable that at least a portion of the larval development is planktonic and dispersal widespread.

Because there appears to be some degree of nonrandom distribution and nonrandom copulations between morph types, it is possible that selection could act to fix these shell characters. However, given microhabitat variability, temporal variation in wave exposure conditions, and evidence for a juvenile planktonic stage, we suspect that shell thickening may be a phenotypic response to environmental conditions.

Ecological and evolutionary implications

Our predation experiments unequivocally demonstrate that the thick *columellaris* morph is relatively more resistant to predation by shell-crushing organisms. Analogous results have been shown for temperate thaidids and littorinids (Kitching *et al.*, 1966; Kitching and Lockwood, 1974; Raffaelli, 1978; Dudley, 1980; Vermeij, 1982). Kitching *et al.* (1966) found that thick forms of *Nucella* predominated along wave protected shores where crab densities were high. Experiments in the laboratory and field showed significantly higher mortality by crab predation among thin compared to thick shells.

Predation rates appear to be low along exposed shorelines perhaps because predators may have a more difficult time negotiating in a high energy environment where swift currents and low visibility could reduce effective foraging. Thin shells therefore may have a refuge from predators in such habitats.

What potential advantage might thin-shelled morphs have over thick-shelled counterparts? Work by Kitching *et al.* (1966) demonstrated that thin-shelled snails were better able to adhere to the substrate, especially where wave action was strong. We have found thick *columellaris* shell morphs more easily dislodged from rocks than thin-shelled *pansa* morphs. The wider aperture and lower spire of the *pansa* morph permit a greater surface area for pedal adhesion. The shells of *Purpura* from very exposed situations tend to be nearly as wide as long giving them a limpet-like appearance. Such animals are extremely difficult to dislodge by hand.

Variation in shell morphology among eastern Pacific *Purpura* may represent an adaptive response to two different and opposing selective pressures; thick shells may be favored where predation risk is high (*i.e.* wave protected shores) and thin shells in environments subject to high wave action but lower predation. The absence of complete segregation of morph types probably reflects the spatial variability in these microhabitats. Snails on the lee sides of large boulders in wave exposed areas are potentially accessible to fish predation.

Since the appearance of the Panamanian isthmus three million years ago (Keigwin, 1978), *P. columellaris* in the eastern Pacific has diverged morphologically while

P. patula has remained conservative: there is no equivalent thick morph counterpart in Caribbean populations. Although, heavy high-spired *P. patula* with thick outer lips are occasionally recovered, these specimens lack any evidence of tooth development (Vermeij, *in* litt.). This dichotomy may be due to differences in predation intensity between oceans. Major differences in shell architecture and ornamentation among gastropod taxa in different oceans have been argued to represent adaptations to gradients in predation intensity (Vermeij, 1976). Laboratory experiments have shown that when eastern Pacific fish (*Diodon* spp.) were presented with eastern Pacific and Caribbean congeners of closely related gastropod species they were able to crush and consume larger-sized Caribbean forms, indicating that snails from this region are less predator resistant (Palmer, 1979 and Palmer cited in Vermeij, 1978 p. 123). Using empty shells in a controlled field experiment, Glynn (unpubl. data) found that the eastern Pacific corallivore, *Jenneria* was heavily preyed on in the eastern Pacific but largely ignored in the Caribbean. Finally, fish predation on hermit crabs occupying gastropod shells was shown to be much greater along the Pacific coast of Panamá than on the Caribbean side of the isthmus (Bertness, 1982).

In addition to faunal differences between oceans, one possible explanation for the apparent low predation rates in the Caribbean, especially for intertidal species, is the small tidal amplitude. In the Bay of Panamá tides flucuate *ca.* 7m but on the Caribbean side it is less than one meter (Glynn, 1972). Hay (pers. comm.) notes that in the Caribbean herbivorous fish are largely excluded from the shallow reef-flat. Possibly predatory fish are also excluded from shallow rocky intertidal areas.

In conclusion, the isolation of conspecific *Purpura* following the appearance of the Panamanian isthmus three million years ago provided a unique natural experiment. The results support Vermeij's hypothesis that interoceanic differences in gastropod shell morphology represent an evolutionary response to differences in predation pressures.

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CHEMOTAXIS OF OYSTER DRILLS *UROSALPINX CINEREA* TO COMPETING PREY ODORS

LESLIE G. WILLIAMS,[1] DAN RITTSCHOF,[2] BETSY BROWN[3]
AND MELBOURNE R. CARRIKER

University of Delaware, College of Marine Studies, Lewes, DE 19958

ABSTRACT

Response of newly hatched, predatory snails to competing chemical cues from co-occurring species of prey were determined in laboratory experiments. Egg capsules of *Urosalpinx cinerea* were collected from an intertidal, vertically zoned community of prey species. The most prevalent prey were barnacles *Semibalanus balanoides* and *Balanus eburneus,* oysters *Crassostrea virginica,* mussels *Mytilus edulis,* and bryozoans *Membranipora tenuis* and *Schizoporella irrorata.* Snail chemotaxis evoked by barnacle odor alone and barnacle odor mixed with odor of either mussels or oysters was assayed in an activity chamber. Mussel odor inhibits chemotaxis to barnacles, but does not evoke chemotaxis itself. Oyster odor inhibits chemotaxis to high concentrations of barnacle odor, but increases chemotaxis to low concentrations of barnacle odor. Chemotaxis to barnacle odor is reduced by 2 h pre-exposure to either barnacle, oyster, or mussel odors. Such cross-adaptation suggests that inhibition or facilitation of chemotaxis in odor mixtures is not caused by one odor masking a second while free in the sea water. We infer that newly hatched snails integrate chemical information in barnacle-oyster and barnacle-mussel odor mixtures. These results, and those of earlier investigations, suggest a behavioral explanation for vertical distribution of juvenile *U. cinerea* in the intertidal zone.

INTRODUCTION

Predators that actively search for food usually show some degree of specificity in what they will and will not eat. Specificity depends on a fundamental aspect of animal behavior, the ability to integrate a spectrum of sensory information and respond only to those cues that are meaningful. Previous studies in our laboratory have shown that balanoid barnacles produce a potent chemical cue that evokes search behavior in both newly hatched and adult *Urosalpinx cinerea* (Say) (Rittschof *et al.,* 1983). *Crassostrea virginica* (Gmelin), however, produce a stimulus that is weakly attractive to newly hatched *U. cinerea* (Rittschof *et al.,* 1983) but is strongly attractive to those snails that have fed upon *C. virginica* (Wood, 1968; Wood *et al.,* 1983). *Mytilus edulis L.* do not seem to evoke chemotactic search behavior at all, though they constitute a major source of prey for *U. cinerea* in some areas (Wood, 1968). This study examines behavioral integration of competing chemical cues by measuring chemotactic orientation of the predatory snail *U. cinerea* to mixtures of prey odor.

Urosalpinx cinerea is a shell-boring, muricid gastropod endemic to the Atlantic Coast of North America (Carriker, 1955). Throughout its range *U. cinerea* occupies rocky and shellbed habitats below the mid-tidal line where it preys upon numerous

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[1] Present Address: Department of Botany KB15, University of Washington, Seattle, WA 98195, [2]Duke University Marine Labs, Pivers Island, Beaufort, NC 28516, and [3]Battelle Marine Research Laboratory, 397 Washington St., Duxbury, MA 02332.

species of sessile, shelled, and encrusting invertebrates, many of which are commercially valuable. The list of prey includes at least 20 species of bivalves, gastropods, barnacles, bryozoans, and small decapod crustaceans (Carriker 1955; Hancock, 1959; Franz, 1971; Pratt, 1974; and Ordzie and Garofalo, 1980). In contrast to the diversity of prey species attacked throughout its range, *U. cinerea* tends to be a stenotrophic predator within its microhabitat, detecting and selecting preferred species of prey by odor (Cole, 1944; Haskin, 1950; Hancock, 1959; Wood, 1968; Pratt, 1974; Ordzie and Garofalo, 1980; Rittschof *et al.,* 1983). In general, prey preference by *U. cinerea* is hierarchical, headed by balanoid barnacles and followed in order by oyster spat and mussels. Finally, *U. cinerea* has also evolved the capability of switching to other species of prey when its preferred prey becomes rare or is no longer available (Wood, 1968). Wood (1968) concludes that prey switching is a result of: 1) population density and distribution of prey, and 2) recent ingestive experience of the predator (*i.e.,* ingestive conditioning).

The role of chemoreception in orientation to prey, prey preference, and prey switching behaviors is complex. Chemotaxis of *Urosalpinx cinerea* toward several, but not all, prey species is an innate behavior. Newly hatched, and ingestively naive, *U. cinerea* are attracted to odors emanating from young hardshell clams [*Mercenaria mercenaria* (L.)], barnacles [*Semibalanus balanoides* (L.) and *Balanus eburneus* Gould], bryozoans [*Schizoporella irrorata* (Watess) and *Membranipora tenuis* Desor], an oyster [*Crassostrea virginica* (Gmelin)], and the tube dwelling polychaete *Sabellaria vulgaris* Verill (Carriker, 1957; Rittschof *et al.,* 1983). Adult *U. cinerea* can chemolocate prey species such as the bay scallop *Argopecten irradians* (Lamarck) that do not evoke chemotaxis in newly hatched snails (Ordzie and Garofalo, 1980; Rittschof *et al.,* 1983). Several species of prey such as slipper limpets *Crepidula fornicata* (L.) and mussels *Mytilus edulis* are not chemotactically located from a distance by either newly hatched or adult *U. cinerea* (*c.f.,* Pratt, 1974 and Rittschof *et al.,* 1983), though both species are attacked and eaten by the snails. In the case of *C. fornicata,* Pratt (1974) further demonstrated that attack (*i.e.,* shell penetration and feeding) was evoked by a chemical cue.

The purpose of this investigation was to determine how *Urosalpinx cinerea* behaviorally integrates competing chemical cues from several co-occurring species of prey, barnacles (*Semibalanus balanoides* and *Balanus eburneus*), oysters (*Crassostrea virginica*), and mussels (*Mytilus edulis*). Better understanding of integration of competing chemical cues is important for further insight into: 1) prey preference and switching behaviors; 2) prey location in an often turbulent aquatic environment where odors of several spatially separate prey species may be mixed; and 3) unique aspects of orientation to prey, though the overall behavioral response, predation, is the same. We addressed the problem by measuring the effects that oyster and mussel odors have on chemotaxis of *U. cinerea* to barnacle stimulus. This was done by comparing snail chemotaxis evoked by individual species odors with that evoked by odor mixtures. Effects of odor mixtures on snail chemotaxis were further quantified by measuring: 1) adaptation of *U. cinerea* to single odor on later response to that odor; and 2) adaptation to one odor on response to a second odor (*i.e.,* cross-adaptation).

Materials and Methods

Collection and culture of Urosalpinx cinerea

Egg capsules of *Urosalpinx cinerea* were collected near mean low water (MLW) from the inner breakwater of Delaware Bay at Lewes, Delaware (38° 47′ N, 75° 06′

W). Capsules were transported to the laboratory in sea water, cleaned of debris by washing on a 3 mm mesh screen, and incubated in filtered sea water as described by Rittschof *et al.* (1983). Newly hatched *U. cinerea* were removed from culture dishes several times a week, and were usually used for experiments within a day of collection. Experimental snails were used only once and therefore were naive in the sense that they neither fed upon nor were exposed to prey prior to an experiment.

Field observations

In August and September, 1980, careful observations were made of the intertidal zone from the site of egg-capsule deposition (about 0.5 m below MLW) to the approximate upper limit of distribution of the barnacle *Semibalanus balanoides* (about 3 m above MLW). Relative position of various potential prey of *Urosalpinx cinerea* was noted, and the prey and substrata searched for recently hatched snails.

Prey species and odor preparation

The four prey species used were *Mytilus edulis, Crassostrea virginica, Semibalanus balanoides,* and *Balanus eburneus.* The barnacles and mussels were collected from the inner breakwater at the time of collection of *Urosalpinx cinerea* egg-capsules. Oysters were removed from a raft culture maintained in the Broadkill estuary, Lewes, Delaware. Specimens were brought into the laboratory, placed in small aquaria (5–40 liters), aerated, and supplied with filtered (0.5 μm) sea water (about 32‰). These animals were used within a week of capture to prepare odor-bearing water for our various experiments. Animals not used within a week of capture were caged in the Broadkill estuary where they could be retrieved for later experimentation.

Odor was prepared by placing unfouled specimens of each prey species into separate 5 liter aquaria containing filtered sea water. Aquaria were bubbled with air overnight. Water was siphoned from each aquarium, filtered through a glass fiber filter (Whatman GF/F), and in some instances refiltered through polycarbonate membrane filters (0.4 or 0.2 μm, BioRad, Inc.). In some instances the stimulus water was frozen in 100 ml polycarbonate containers and thawed prior to use. Stimulus preparation and determination of stimulus potency is reported in more detail by Rittschof *et al.* (1983).

Bioassay procedure

Assay Device. Distance chemotaxis was measured in an assay device consisting of a reservoir of stimulus solution held in a 500 ml mariotte flask, a 2 ml calibrated pipette bent into an elbow at one end, and an intervening flow meter (Gilmont, Inc.). Four to six devices were run simultaneously. During each experiment, 15 to 30 newly hatched snails were transferred from 20 ml vials into the open mouth of each pipette with a small paint brush, and allowed 30 s to attach to the interior surface of the pipette. Stimulus flow was then started and adjusted to the optimum discharge rate of 7.5 ml/min (approximately 75 cm/min linear velocity). Further details of methods are reported by Rittschof *et al.* (1983).

Chemotaxis. The assay required an all-or-none type of response from the snails. The response criterion chosen was upstream movement of at least 1 cm within 10 min after restarting the stimulus flow (Rittschof *et al.,* 1983). Since homogeneous stimulus solutions were used in this study, there was no chemical gradient to direct the snails' responses. Responses to the various chemical cues were determined by comparison of their active upstream migration in odor-laden currents of water with their relative inactivity under identical, odor-free conditions of flow. Therefore, the behavior assayed was a non-random, chemically stimulated rheotaxis, which will be referred to as chemotaxis in this paper.

Mixed stimulus experiments

Mussel-Barnacle. In two experiments, we compared chemotaxis of *Urosalpinx cinerea* to barnacle odor (*Balanus eburneus*) with that evoked by a mixture of barnacle and mussel odors. The same stimuli were used in each experiment. Barnacle odor was prepared ahead of time by filtration (0.4 μm), and rapid freezing of 100 ml aliquots on dry ice. Mussel odor was prepared fresh by filtering it through Whatman GF/F and 0.4 μm polycarbonate filters, and cooling the filtrate on ice until needed. We then measured chemotaxis of *U. cinerea* to a range of dilutions of barnacle stimulus in order to establish expected frequencies of response for the subsequent mixed odor experiments. Chemotaxis evoked by 1.0% and 5.0% (v/v) dilutions of barnacle stimulus in each of a set of four dilutions of mussel stimulus (10.0, 5.0, 1.0, and 0.5% v/v and 10.0, 4.0, 1.0, and 0.1% v/v respectively) was measured. Stimulus strength was calculated as the percentage of volume of stimulus water in the total volume of solutions.

Oyster-Barnacle. Two series of experiments were conducted to assess the effect of oyster odor on the chemotactic response of *Urosalpinx cinerea* to barnacle odor. In the first series, three experiments were performed to establish the snails' response to various dilutions of: 1) barnacle odor alone (*Semibalanus balanoides;* 10.0, 2.0, 1.0, and 0.5%); 2) oyster odor alone (100, 10.0, 1.0, and 0%) and 3) barnacle odor mixed with 100% oyster odor (10.0, 1.0, 0.5, and 0%). The barnacle odor was the same for each experiment, and was prepared in advance by filtration (0.4 μm) and freezing on dry-ice. Oyster odor was made by placing 5 oysters (total wet wt = 119.1 g) in 3 liters of sea water for 3 h, siphoning the sea water from the aquarium, and filtering it through glass fiber filters (Whatman GF/F).

In the second series, four experiments measured the effects of different concentrations of concurrent oyster odor on the response to barnacle odor (*Balanus eburneus*). The first experiment measured the response to 5.0, 2.0, 1.0, and 0.7% dilutions of barnacle odor mixed in 95% oyster odor. The second, third, and fourth experiments measured the response to the four dilutions of barnacle odor mixed in 10, 1.0, and 0.1% oyster odor respectively. The same batch of filtered and frozen barnacle stimulus was used in each experiment, and was prepared as described for the mussel-barnacle experiments. Oyster stimulus was prepared by allowing 8 oysters to pump in an aquarium containing 5 liters of filtered sea water for 3 h. The water was siphoned from the aquarium, filtered (GF/F), and cooled in an ice bath.

Adaptation experiments

This experiment examined the effects of prolonged exposure to *Semibalanus balanoides* odor on chemotaxis of newly hatched *Urosalpinx cinerea.* Barnacle odor, which had been filtered and frozen, was thawed, diluted, and warmed to 22°C immediately before use in this experiment. Snails were apportioned into 2 groups of about 300 snails each, and caged in 2 20 ml plastic vials. The vials were screened at both ends to permit circulation of sea water. One vial was submerged in a 10% dilution of barnacle stimulus and the second in filtered sea water (GF/F). After 2 h, a sample of snails from each treatment was assayed in a dilution series of barnacle stimulus (10.0, 1.0, 0.5, and 0%).

Cross-adaptation experiments

Two experiments were performed to test the effects of 2 h pre-exposure to oyster and mussel odors on chemotaxis to barnacle (*Balanus eburneus*) odor. In the first experiment, snails were divided into groups of 300 snails, and each group caged in a screened, 20 ml vial. Vials were then submerged for 2 h in either undiluted oyster stimulus, mussel stimulus, barnacle stimulus or filtered sea water. Samples of snails from each vial were then assayed for their response to 1% and 10% barnacle odor.

Lastly, the effect of 2 h pre-exposure to filtered sea water, oyster, mussel, and barnacle odors on the response to 10% dilutions of either oyster or mussel odors was determined. The various odors for these experiments were filtered (GF/F), frozen, and stored at −20°C. Prior to each experiment, the frozen samples were thawed, filtered (0.4 μm), and diluted, when appropriate, with similarly filtered sea water.

Statistics

The effect of stimulus dilution on chemotaxis was tested for significance by means of the log-likelihood ratio or G-test (Sokal and Rohlf, 1969). Data were further analyzed by calculating the proportion (P) of snails responding to each dilution, and then by regression of the angle of P (angle = arcsin of the square root of P) on the log of stimulus dilution. The angular transformation is helpful in simplifying a curvilinear relationship into a linear one, particularly where very high (>70%) or very low (<30%) frequencies of response occur.

RESULTS

Field Observations

The inner breakwater of Delaware Bay is vertically zoned with respect to distribution of potential prey of *Urosalpinx cinerea. Semibalanus balanoides* occurs in the upper intertidal and intergrades with the top of the upper end of the *Mytilus edulis* zone at about 50 cm above mean low water (MLW). A small population of *Crassostrea virginica* occupies a narrow zone 40–60 cm above MLW. Bryozoans, chiefly *Membranipora tenuis* and *Schizoporella irrorata,* occur about 50 cm below MLW, at about the same elevation as egg capsules of *U. cinerea.* Newly hatched snails (less than 2 cm axial length) were observed below MLW at the bases of egg capsules, upon the shells of adult *U. cinerea,* and upon barnacle, mussel, and bryozoan prey. Adult snails were found as high as 100 cm above MLW.

Mixed stimulus experiments

Mussel-Barnacle. Mussel odor inhibits the response of *Urosalpinx cinerea* to odor of *Balanus eburneus.* In each of two experiments conducted, the response of newly hatched snails to barnacle odor alone was significantly associated with stimulus dilution ($P < 0.005$). Data pooled from these experiments show an increase in frequency of response to barnacle odor as a function of increasing stimulus strength (Fig. 1). This relationship predicts 33% and 15% responses to 5% and 1% dilutions of barnacle odor respectively. Response to 1% barnacle odor mixed with varying concentrations of mussel odor began to decrease significantly ($P < 0.005$) from the predicted 15% response with increasing concentration of mussel stimulus (Fig. 2). Response to 5% barnacle odor mixed with mussel odor also declined significantly ($P < 0.05$) below the 33% predicted response.

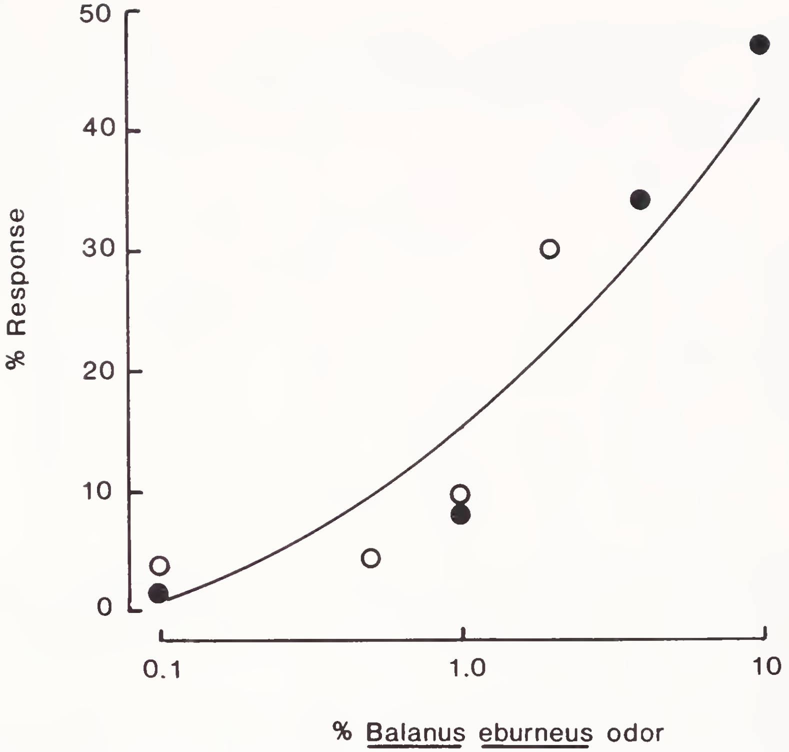

FIGURE 1. Frequency of reponse of newly hatched *Urosalpinx cinerea* to *Balanus eburneus* odor (BEO) plotted as a function of stimulus dilution. The curve was fitted from the regression equation

$$\text{Arcsin } P = 17.6 \log(\text{BEO}) + 58.1$$

with a coefficient of determination of 0.842. P = the proportion of snails responding. Open and closed symbols represent experiments performed several hours apart. Sample size for each data point ranged from n = 64 to n = 81 snails.

Oyster-Barnacle. Chemotaxis of newly hatched snails to either barnacle (*Semibalanus balanoides*) odor, oyster odor, or a mixture of the two was significantly associated with stimulus dilution in each case ($P < 0.05$). Frequency of response increased with increased strength of barnacle and oyster stimuli. However, both rate of increase and magnitude of response was greater for snails exposed to barnacle odor than for those exposed to oyster odor. Response to dilutions of barnacle odor mixed with 100% oyster odor was intermediate, both in magnitude and in rate of increase, to that evoked by barnacle or oyster odors individually (Fig. 3). In the second series of four experiments that measured response to a range of concentrations of barnacle (*Balanus eburneus*) odor mixed in various dilutions of oyster odor (95, 10, 1.0, and 0.1%), the overall chemotactic response was dominated, as expected, by barnacle odor ($G = 80.06$, $P < 0.005$). However, oyster odor had a significant effect on chemotaxis at 3 of the 4 concentrations of barnacle odor (Table 1). Oyster odor inhibited the response evoked by 5% barnacle odor, and either facilitated or remained neutral to the response evoked by lower dilutions of barnacle odor.

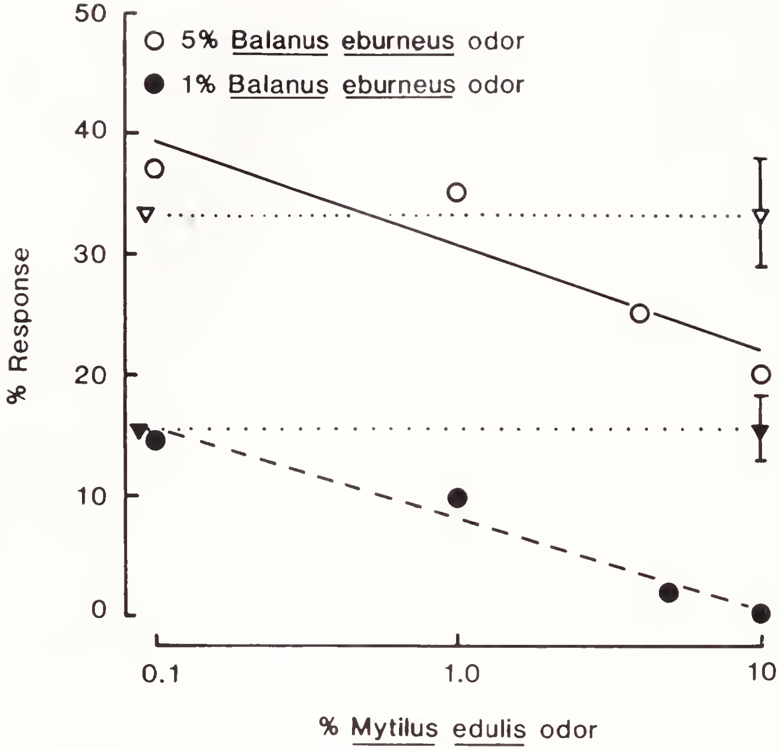

FIGURE 2. Response of *Urosalpinx cinerea* to a mixture of *Balanus eburneus* and *Mytilus edulis* odors, and plotted as a function of *M. edulis* stimulus dilution. Inverted triangles and dotted lines show the % response (±1 standard deviation) predicted from the control experiment shown in Figure 1. Negative slope of lines fitted to data indicate that *M. edulis* inhibits chemotaxis to *B. eburneus*. Sample size at each dilution of *M. edulis* odor ranged from n = 67 to n = 81.

Adaptation experiment

Chemotaxis of *Urosalpinx cinerea* was significantly associated with dilution of *Semibalanus balanoides* odor ($P < 0.005$) as well as with prior exposure to barnacle odor ($P < 0.005$). As hypothesized, pre-exposure to barnacle stimulus decreased the response of the snails, but only at the lower concentrations of barnacle odor (Fig. 4). Pre-exposure to 10% barnacle odor did not significantly affect response of the snails to either 10% barnacle odor or filtered sea water (*i.e.*, rheotaxis).

Cross-adaptation experiments

There was a highly significant ($P < 0.005$), concentration dependent association between chemotaxis of snails to barnacle odor and their prior exposure to filtered sea water, or oyster, mussel, and barnacle odors. As anticipated in the foregoing adaptation experiments, chemotaxis to barnacle odor was least for snails exposed to filtered sea water. This generalization is true for snails responding to either 10% or 1% barnacle odor, the only difference being in magnitude of response (Fig. 5).

TABLE I

Contingency table summarizing the effects of Crassostrea virginica *odor (CVO) on chemotaxis of* Urosalpinx cinerea *to each of 4 concentrations of* Balanus eburneus *odor (BEO)*

Exp.	BEO (%)	CVO (%)	Response	N	% Response	Overall-G	*P*
1	5.0	95	29	66	44		
1	5.0	10	37	61	61		
1	5.0	1.0	63	94	67		
1	5.0	0.1	44	66	67	10.14	<0.005
2	2.0	95	30	42	71		
2	2.0	10	27	63	43		
2	2.0	1.0	40	88	45		
2	2.0	0.1	28	63	44	10.63	<0.010
3	1.0	95	16	64	25		
3	1.0	10	10	55	18		
3	1.0	1.0	33	90	37		
3	1.0	0.1	22	61	36	7.68	>0.050
4	0.7	95	21	49	43		
4	0.7	10	25	71	35		
4	0.7	1.0	18	74	24		
4	0.7	0.1	9	62	15	13.53	<0.005

Stimulus strength is expressed as a percentage of the volume of stimulus water in the total volume of stimulus solution. Tests for significance of association between chemotaxis and strength of oyster odor are summarized as Overall-G for each concentration of barnacle odor.

Snails pre-exposed to oyster or mussel odors showed greater response to barnacle odor than those pre-exposed to barnacle odor, but showed a lower response than those pre-exposed to filtered sea water.

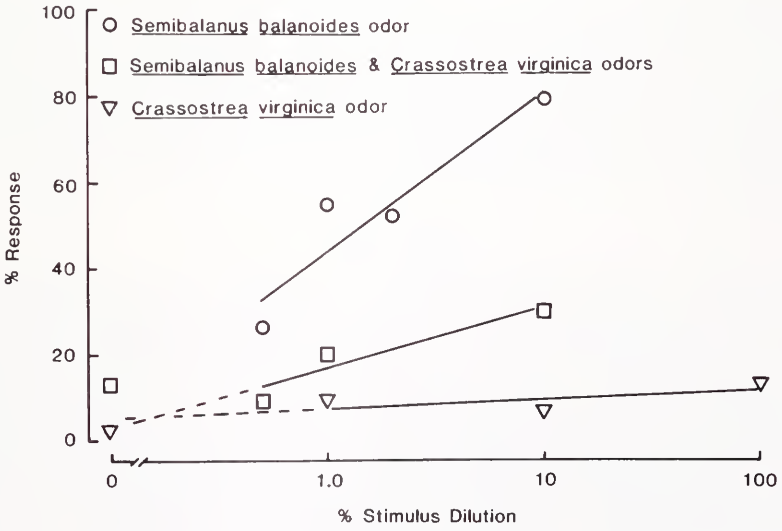

FIGURE 3. Response of Urosalpinx cinerea to dilutions of mussel or oyster odor, or to a mixture of the two. Lines fitted by least squares regression of % response on log of stimulus dilution. Zero (*i.e.*, control) values were omitted from the regression because such values are incompatible with the logarithmic transformation. Sample size for the various data points ranged from n = 61 to n = 71 (*Semibalanus balanoides*), n = 67 to n = 73 (*Crassostrea virginica*), and n = 55 to n = 68 (*S. balanoides* + *C. virginica*).

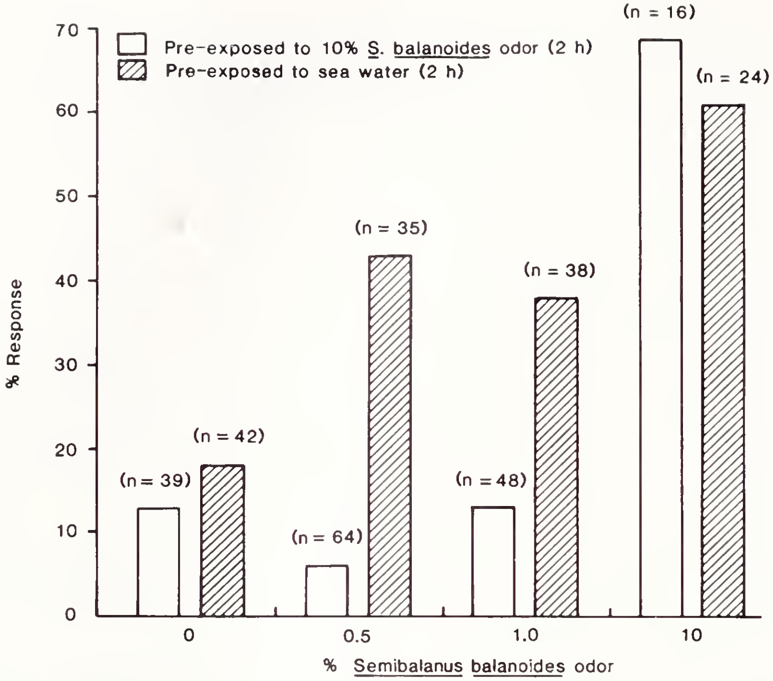

FIGURE 4. Effect of 2 h exposure to either 10% *Semibalanus balanoides* odor or sea water on chemotaxis of *Urosalpinx cinerea* to barnacle odor. Sample size for each treatment shown in parentheses.

Cross-adaptation effects are less clear in the case of response to mussel or barnacle odors following pre-exposure of snails to sea water, or barnacle, mussel, and oyster odors (Fig. 6). Chemotaxis to 10% oyster odor was equally and significantly ($P < 0.05$) depressed for snails pre-exposed to the 3 odors. Chemotaxis to 10% mussel odor was unaffected by pre-exposure to barnacle or mussel odors, but depressed by pre-exposure to oyster odor (Fig. 6).

DISCUSSION

Our mixed prey experiments reaffirm the chemotactic potency of barnacle odor for newly hatched *Urosalpinx cinerea,* and further show that chemical cues emanating from *Mytilus edulis* and *Crassostrea virginica* significantly affect the chemotactic response of *U. cinerea* to barnacle odor. Moreover, concentration-dependent differences in chemotaxis to the various mixtures suggest qualitative differences among the three stimuli. Mussel odor alone does not evoke chemotaxis, but inhibits response to all concentrations of barnacle odor. Oyster odor inhibits the response to high concentrations of barnacle odor but facilitates the response to low concentrations of barnacle odor. It must be emphasized that the response to oyster odor mixed with low concentrations of barnacle odor (0.7%) exceeded that anticipated to oyster odor alone (*i.e.,* about 20% maximally). This observation suggests that the interaction of oyster and barnacle odors at these concentrations may be additive or synergistic (Fig. 3, Table I).

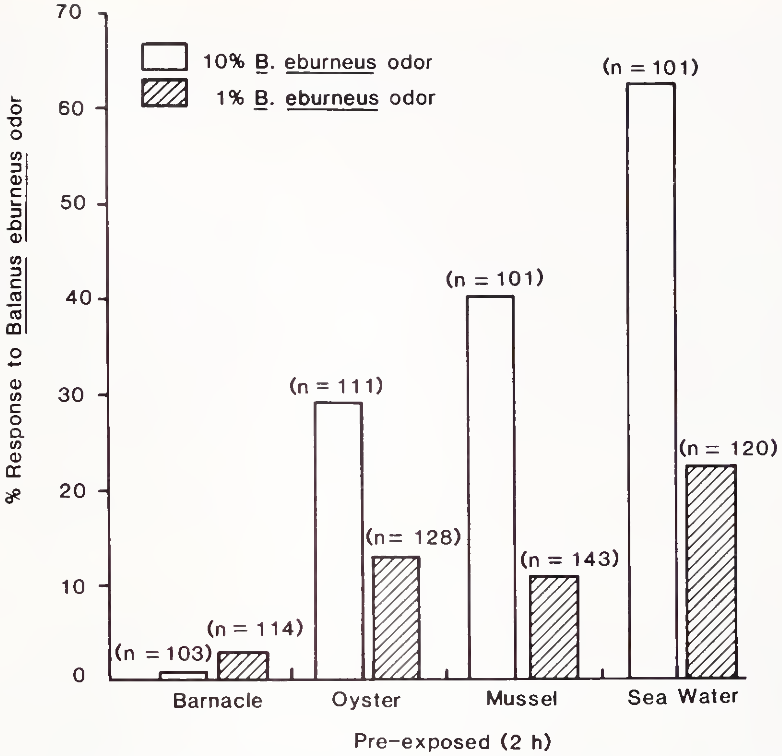

FIGURE 5. Cross-adaptation experiment. Chemotaxis of newly hatched snails to 1% and 10% barnacle odor following 2 h pre-exposure to *Balanus eburneus*, *Crassostrea virginica*, or *Mytilus edulis* odors or to sea water. Sample size shown above bars in parentheses.

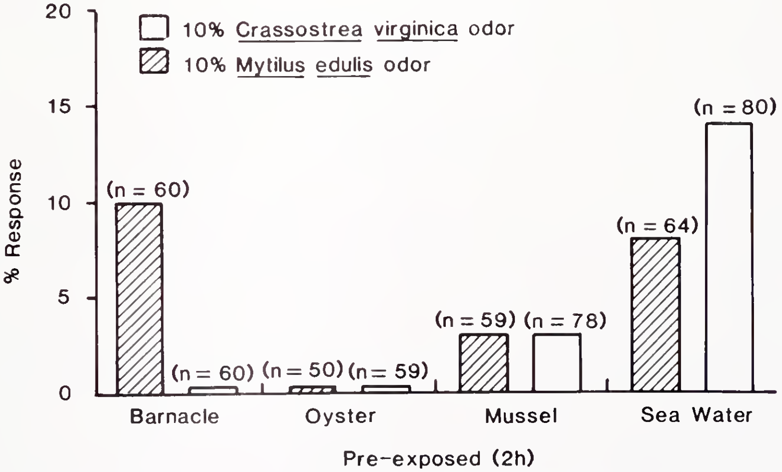

FIGURE 6. Chemotaxis of newly hatched *Urosalpinx cinerea* to 10% oyster and mussel odors following 2 h pre-exposure to either filtered sea water, or *Balanus eburneus*, *Crassostrea virginica*, or *Mytlius edulis* odors. Sample size shown above bars in parentheses.

If the three odors are chemically the same, we would expect reduced chemotaxis to oyster and mussel odors following pre-exposure of snails to barnacle odor. However, pre-exposure to barnacle odor inhibited chemotaxis to oyster odor but not to mussel odor (Fig. 6). Also, we would not expect inhibition (*i.e.,* cross-adaptation) of chemotaxis to a strong barnacle stimulus (*i.e.* one that evokes a 60% response, Fig. 5) following pre-exposure to weak oyster or mussel odors, neither of which evokes more than a 20% response. Clearly, these weak chemotactic odors are capable of inhibiting the response to the much more powerful barnacle odor, again suggesting that the three are indeed different. These results suggest that the effects of stimulus mixtures on chemotaxis are perceptual or behavioral, and not a result of one stimulus masking a second while free in the sea water. Perceptually, cross-adaptation could be explained by any one of several mechanisms; sensory adaptation, sensory fatigue, or stimulus filtering (Hinde, 1966). Similarly, at the behavioral level, cross-adaptation could be explained by response specific fatigue (Manning, 1979), or by generalized habituation to several stimuli (Petrinovich, 1973; Wyers *et al.,* 1973). However, habituation is an unsatisfactory explanation of snail chemotaxis in odor mixtures because the 10 min period of our bioassay is too brief to bring it about (LGW and BB, unpublished observations).

More importantly, results concerning response to odor mixtures offers new insight into mechanisms of orientation, location, and preference of prey. Wood (1968) and Wood *et al.* (1983) demonstrated that odor of *Crassostrea virginica* may become as attractive as odor of *Balanus* spp to *Urosalpinx cinerea,* but only if *U. cinerea* had previously fed upon *C. virginica* for a period of time. Wood (1968) speculated that this change in response to oyster odor could be a form of associative learning if a novel olfactory stimulus were paired with reinforcement offered by ingestion of oyster flesh. From this study as well as that of Wood (1968), Wood *et al.* (1983), and Rittschof *et al.* (1983), it seems that the criterion of simultaneous olfactory stimulation by a novel stimulus associated with food has been satisfied. This type of associative process could also account for the switching behavior of another predatory, shell-boring gastropod, *Acanthina spirata* (Blainville) (Murdoch, 1969). This type of olfactory plasticity appears quite common and has been identified in orientation to prey by terrestrial snails (Croll and Chase, 1980) and tuna (Atema *et al.,* 1980), and in orientation to host symbionts by pea crabs (Derby and Atema, 1980) and scale worms (Dimock and Davenport, 1971).

Lastly, we hypothesize that vertical migration of newly hatched snails is affected by prey odors primarily through interaction with one another and with abiotic factors (water current and gravity). Egg capsules in newly hatched snails occur subtidally, near MLW, 100 cm or more below the upper (mid-tidal) end of distribution of the adult snails and barnacle prey. Carriker (1957) observed that geotaxis of young snails is dependant on their state of hunger; unfed snails move upward while those that are satiated or exposed to food do not. Rheotaxis reported in this study and by Rittschof *et al.* (1983) is motivated by hunger and released by chemical cues associated with prey. Furthermore, rheotaxis stimulated by low concentrations of barnacle odor may be inhibited or out-competed by high concentrations of mussel or oyster odor. Thus, proximity or abundance of mussels or oysters, prey species first encountered by newly hatched and upwardly migrating snails, may inhibit or compete with the odor of more distant barnacles thereby arresting further upward migration. The combined effects of chemo-, geo-, and rheotaxes bring newly hatched snails within reach of prey in the lowest possible part of the mid-tidal region. Bertness (1977) and Butler (1979) have postulated similar integration of geotactic, photoorthokinetic, and predatory behaviors to account for the vertical distribution of two other murcids, *Nucella emarginata* and *N. lamellosa* (Gmelin). Clearly, behavioral integration of a complex mixture of abiotic cues and prey odors is an important aspect of the distribution and ecology of *Urosalpinx cinerea* and related predatory boring gastropods.

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A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in WORLD LIST *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

F. All single word journal titles in full (*e.g. Veliger, Ecology, Brain*).

G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some BIOLOGICAL ABSTRACTS listings).

H. A few well-known international journals in their preferred forms rather than WORLD LIST or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond., Science, N.Y.; Evolution, Lancaster, Pa.*)

6. ***Reprints, charges.*** *The Biological Bulletin* has no page charges. However, authors will be requested to help pay printing charges of manuscripts that are unusually costly due to length or numbers of tables, figures, or formulae. Reprints may be ordered at time of publication and normally will be delivered about two to three months after the issue date. Authors (or delegates or foreign authors) will receive page proofs of articles shortly before publication. They will be charged the current cost of printers' time for corrections to these (other than corrections of printers' or editors' errors).

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